Universidade de Aveiro Departamento de Biologia
Ano 2015

Ângela Patricía da Silva Correia

O papel do prião celular na doença de Alzheimer
The role of cellular prion protein in Alzheimer's disease

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The role of cellular prion protein in Alzheimer's disease

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia molecular e celular realizada sob a orientação científica da Doutora Etelvina Figueira, Professora auxiliar do Departamento de Biologia da Universidade de Aveiro.

À memória da minha avó Teresa.

o júri

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palavras-chave Prião celular, Alzheimer, beta amilóide, comportamento

resumo

Doença de Alzheimer (AD) é a doença neurodegenerativa mais comum relacionada com a idade que conduz à disfunção cognitiva e demência. A principal característica patológica da doença de Alzheimer é definida pela acumulação de beta-amilóide (Aβ), um péptido neurotóxico, derivado da clivagem da proteína precursora da amilóide (APP) pela beta e gama-secretase. Embora tenha sido descrito que o prião celular (PrP^C) desempenha um papel na patogénese da doença de Alzheimer, o seu papel ainda é pouco claro. Diversos estudos têm mostrado resultados contraditórios em relação à sua função na doença de Alzheimer. Para esclarecer esta questão, o principal objetivo deste estudo é investigar a influência do gene *PRNP* em ratinhos 5xFAD. Os ratinhos 5xFAD exibem 5 mutações envolvidas na doença de Alzheimer familiar. Estes apresentam a acumulação de Aβ1-42 e um aumento da perda neuronal durante o envelhecimento. Para criar ratinhos bi-transgénicos, os ratinhos 5xFAD foram cruzados com $Prnp^{0/0}$ Zurich 1 (nocaute para o prião celular). Os ratinhos transgénicos (5xFAD e $Prnp^{0/0}$5xFAD) com diferentes idades (3, 9 e 12 meses de idade) foram submetidos a uma bateria de testes de avaliação cognitiva e motora. Em seguida procedeu-se a uma avaliação bioquímica (ELISA, western blot e imunohistoquimica) para investigar o potencial envolvimento do PrP^C na regulação e sinalização proteica induzida pela toxicidade da Aβ. O estudo revelou que os défices induzidos pela toxicidade da Aβ aparecem mais cedo nos ratinhos 5xFAD (9 meses de idade) do que nos $Prnp^{0/0}$5xFAD (12 meses de idade). A produção de beta amilóde revelou uma regulação dependente do PrP^C na isoforma Aβ1−40 ao contrário da isoforma Aβ1−42. Ao contrário do que acontece nos ratinhos 5xFAD, não foi encontrada nenhuma sobre expressão das proteínas P-Fyn, Fyn e Cav-1 nos ratinhos $Prnp^{0/0}$5xFAD. Estes resultados sugerem um papel importante do PrP^C na doença de Alzheimer como promotor do efeito tóxico dos oligómeros de beta amilóide, uma vez que a perda do PrP^C atrasa o efeito tóxico dos oligómeros de beta amilóide. Em conclusão, os nossos dados apoiam o papel do PrP^C como um agente mediador de toxicidade da Aβ na doença de Alzheimer. PrP^C promove o início precoce da doença de Alzheimer.

keywords Cellular prion protein, alzheimer, behavior, beta amyloid

abstract

Alzheimer's disease (AD) is the most prevalent age-related neurodegenerative disease that leads to cognitive impairment and dementia. The major defined pathological hallmark of AD is the accumulation of amyloid beta (Aβ), a neurotoxic peptide, derived from beta and gamma-secretase cleavage of the amyloid precursor protein (APP). It has been described that cellular prion protein (PrP^C) plays a role in the pathogenesis of Alzheimer disease. Although, the role of PrP^C is still unclear, previous studies showed contradictious results. To elucidate this issue, the main objective of the present study is to investigate the influence of a knockout of the PRNP gene in 5XFAD mice, 5xFAD mice exhibited 5 mutations related to familial Alzheimer disease. These mice show an Aβ1-42 accumulation and an increased neuronal loss during aging. To create a bi-transgenic 5xFAD mice were crossed with $Prnp^{0/0}$ Zurich 1 mice (prion protein knockout mice). We subjected two transgenic mice (5xFAD and $Prnp^{0/0}$5xFAD) at different ages (3, 9 and 12 months of age) to a battery of task to evaluate cognitive and motoric deficits and a biochemical analysis (ELISA, western blot and immunohistochemistry) to investigate the regulation and potential involvement of downstream signaling proteins in the Aβ induced toxicity process dependent of the PrP^C concentration. The study revealed that the deficits induced by Aβ mediated toxicity appeared earlier in 5xFAD mice (9 months of age) than in $Prnp^{0/0}$5xFAD (12 months of age). Investigating the amount of amyloid beta in 5xFAD mice we observed a PrP^C dependent regulation in 9 month-old animals of Aβ1−40 but not of the toxic form Aβ1−42. We did not found in $Prnp^{0/0}$5xFAD mice the up-regulation of P-Fyn, Fyn or Cav-1 as we found in 5xFAD mice. This suggests an important role of PrP^C in Alzheimer's disease as a promoter of toxic effect of Aβ oligomers. Our results may suggest the loss of PrP^C delays the toxicity of amyloid beta. In conclusion, our data support a role of PrP^C as a mediator of Aβ toxicity in AD by promoting early onset of disease.

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Abbreviations

5xFAD	5 mutation for AD
aa	Amino acid
AD	Alzheimer's disease
APOE4	Apolipoprotein E4
APP	Amyloid percursor protein
bp	Base pair
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt–Jakob disease
iCJD	Iatrogenic Creutzfeldt–Jakob disease
vCJD	Variant Creutzfeldt–Jakob disease
CNS	Central nervous system
CWD	Chronic wasting disease
ddH2O	Double distilled water
dH20	Distilled water
ECL	Enhanced chemiluminescence
EEG	Electroencephalography
EPM	Elevated Pluz Maze
FAD	Familial Alzheimer's disease
FC	Fear Conditioning
FFI	Fatal familial insomnia
GSS	Gerstmann-Sträussler-Scheinker syndrome
H	Hour
kDa	Kilodalton
LBD	Lewy body disease
M	Mol
mg	Milligram
min.	Minute
ml	Milliliter
NOR	Novel Object Recognition
^{o}C	Celsius
OF	Open field
PD	Parkinson Disease
PBS	Phosphate buffered saline
PRNP	Prion protein gene
$Prnp^{0/0}$	PrPC knockout
$Prnp^{0/0}$5xFAD	PrPC knockout with 5 mutations for AD
PrP^{C}	Cellular prion protein
PrP^{SC}	Infectious isoform of prion protein
PSEN1	Presenilin 1
PSEN2	Presenilin 2

PVDF	Polyvinylidene fluoride
rpm	Rotations per minute
RR	Rotarod
RT	Room temperature
sCJD	Sporadic Creutzfeldt-Jakob disease
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering RNA
TBST	TBS with 0.1% Tween
TE	Tris EDTA
TEMED	N, N, N´, N´-tetramethylethylenediamine

I. Introduction

1. Neurodegenerative Dementias

Neurodegenerative dementias are characterized by an insidious onset that is followed by a gradual and slowly progressive cognitive impairment. Until now there is no cure for neurodegenerative dementias that result in accumulation of proteins in the brain which is followed by a progressive degeneration and/or death of nerve cells. These alterations can lead to multiple cognitive disturbances such as movement problems (ataxia), or mental function problems (dementia). There are many different forms of neurodegenerative dementia and they are histologically characterized by varying degrees of neuronal loss, gliosis, and usually with abnormally misfolded protein depositions (Josephs et al. 2009). The most common form of neurodegenerative dementia is Alzheimer's disease (approximately 55%), followed Lewy body disease (LBD), stroke/mixed dementia and frontotemporal dementia (Figure 1) (Waight et al. 2015).

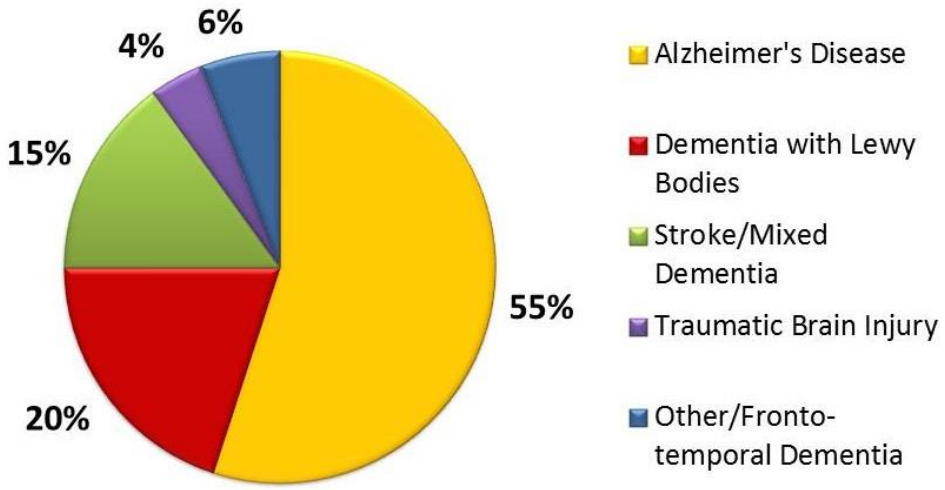

Figure 1. Types of dementia: Alzheimer's disease is the most incident. (Waight et al. 2015)

The protein deposition nature of proteins defines the histological classification of each neurodegenerative dementia in three major groups: tauopathies, amyloidophathies and synucleinopathies, associated with the pathological aggregation of tau, amyloid or alpha-synuclein proteins in the brain, respectively

(Galpern & Lang 2006). The phenotypic variability in neurodegenerative dementia has been investigated and a spectrum of relations between clinical syndromes and molecular features has been identified. Some proteins have emerged as main players in the mechanism of neurodegeneration. However, the molecular events involved in neurodegeneration remains largely unknown (Caberlotto & Nguyen 2014).

Neurodegenerative diseases are strongly correlated with aging. European countries have a high ageing population, wherein about 16% of the European population is over 65 years-old and it is predicted to reach 25% by 2030 (JPND 2014). The prion diseases are rare neurodegenerative diseases, and it is therefore difficult to know the correct prevalence of prion disease. However, studies suggest that about one person per million worldwide each year is affected by prion diseases (Anon 2015).

1.1. Prion diseases

Prion diseases or also called transmissible spongiform encephalopathies (TSEs) are a family of rare progressive neurodegenerative disorders of central nervous system (CNS) that affect humans and animals. Prion diseases are characterized by rapidly progressive neurodegeneration and mortality (Center For Disease Control 2012). Currently, there is no therapy available other than palliation.

Human prion diseases are classified into Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker Syndrome (GSS), Fatal Familial Insomnia (FFI) and Kuru (Table 1). In animals the prion diseases are classified into Bovine Spongiform Encephalopathy (BSE) in cattle, Chronic Wasting Disease (CWD) in North American cervids, scrapie in sheep and goats, transmissible mink encephalopathy, feline spongiform encephalopathy and ungulate spongiform encephalopathy (Center For Disease Control 2012) TSEs are distinguished by long incubation periods (years or even decades), multifocal spongiform changes associated with neuronal loss, astrogliosis and a failure to induce inflammatory response (Collinge & Alpers 2006).

Table 1. Transmissible spongiform encephalophathies (TSEs) in humans (Liemann & Glockshuber 1998).

Disease name	Etiology
Creutzfeldt-Jakob Disease:	
Iatrogenic	Infection
Sporadic	Unknown
Inherited	Mutation in PrP gene
New variant	Infection from bovine prion?
Gerstmann-Stränssler-Scheinker syndrome	Mutation in PrP gene
Fatal Familial Insomnia (FFI)	Mutation in PrP gene
Kuru	Infection

Prion diseases result from misfolding of a normal cell-surface brain protein called prion protein (PrP^C), whose exact function is unknown. Misfolded prion proteins are called prions or scrapie PrP (PrP^{Sc} - It comes from the name of the prototypic prion disease of sheep). PrP^{Sc} are pathogenic and infectious. Prion disease is produces by replication where PrP^{Sc} induces conformational transformation of PrP^C, creating PrP^{Sc} duplicates, which, in chain reactions, induces further transformation of PrP^C into PrP^{Sc} (Figure 2). This transformation process spreads to various regions of the brain (Gambetti 2015). Both isoforms share identical amino acid sequences, but PrP^{Sc} differs biochemically from PrP^C by its β-sheet enriched structure, detergent insolubility and limited sensitivity to proteolysis by proteinase (Prusiner 1998). PrP^{Sc} is widely believed to be the pathogenic agent in prion diseases (TSEs) (Caughey et al. 1991; Pan et al. 1993).

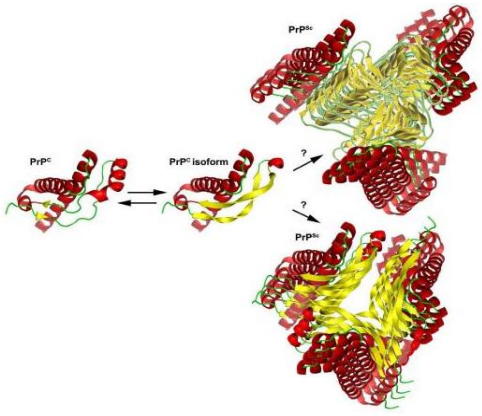

Figure 2. Model of PrP^C isoform and PrP^{Sc}: Representation of PrP^C structure, putative PrP^C isoform, and two PrP^{Sc} models (Samson & Levitt 2011). The proposed structure of PrP^C is composed by 40% of alpha-helix and 3% of β-sheet. When PrP^C is converted to abnormal isoform, PrP^{Sc}, β-sheets

increased to 40% and alpha-helix decrease to 30%. Convertion of PrP^{C} to PrP^{Sc} leads to their accumulation in extracellular and/or intracellular environments (Lee et al. 2013; Moore et al. 2009).

1.1.1. Prions theory

Over the years, researchers have suggested several theories to explain the causative agent of TSEs, called scrapie agent. During several years, many researchers postulated that TSEs were caused by “slow viruses”. However, the researchers were unable to isolate any virus. Subsequent studies revealed that the scrapie agent has many chemical properties in common with a protein molecule (Prusiner 1982; Prusiner et al. 1981). In 1982, Prusiner showed that the scrapie agent is a small proteinaceous infectious particle called the prion (Prusiner 1982). prion describes an abnormal folding of specific normal cellular proteins that are mostly found in the brain, called cellular prion proteins (PrP^{C}) that become resistant to proteases and lead to their accumulation and become able to induce abnormal folding to others PrP^{C} proteins (Center For Disease Control 2012)

1.1.2 Human Prion diseases

The prion diseases in humans are very rare, account approximately 1-2 per 1 million people in worldwide that die due to prion disease each year (Holman et al. 2010). Prion diseases are invariably fatal and incurable becoming a significant concern for animal and human health. Prion diseases can occur sporadically (apparently starting spontaneously, without a known cause), via mutation in *PRNP* (gene of prion protein) and acquired (infectious transmission). The sporadic form of prion disease is the most common form of prion disease. It is estimated that 85% of prion disease are sporadic, 15% are genetic and <1% are acquired (Appleby & Lyketsos 2011; Geschwind 2015).

To explain the occurrence of sporadic CJD two hypotheses have been suggested (Prusiner 1998). The first hypotheses suggests that somatic mutation of prion protein gene (*PRNP*) is correlated with age and this mutation could lead to formation of PrP^{res} (protease resistant prion protein) (Center For Disease Control 2012). The second hypotheses suggests that occurs by the spontaneous conversion

of PrP^{C} into PrP^{res} in a single neuron or a group of neurons, possibly after a chance error during prion protein gene expression (Belay 1999).

The human prion diseases that are associated with autosomal dominant pathogenic mutations in *PRNP* (Gambetti et al. 2003; Collinge 1997; Kovács et al. 2002; Mead 2006). It is believed that the mutation increases the tendency of PrP^{C} to convert into PrP^{Sc} (Riek et al. 1998; Swietnicki et al. 1998). More than 30 autosomal dominant pathogenic *PRNP* mutations have been described (Collinge & Alpers 2006; Wadsworth et al. 2003; Kovács et al. 2002; Mead 2006). It has been shown that the homozygosity for methionine at codon 129 of *PRNP* gene, where either a methionine or a valine may be encoded, represents a risk for development of prion diseases (Palmer et al. 1991).

The Inherited prion diseases are subdivided in GSS, CJD and FFI. Each sub-division is characterized by a specific clinical syndrome, being GSS characterized by the presence of chronic cerebellar ataxia with later pyramidal features and dementia than can be seen in classical CJD (Collinge & Palmer 1997). Fatal familial insomnia (FFI) is characterized by progressive untreatable insomnia, thalamic degeneration, dementia and dysautonomia, it is most commonly associated with a missense mutation at codon 178 of *PRNP* (Medori et al. 1992). However, FFI without causative mutation in *PRNP* has been reported (Masters et al. 1985; Montagna et al. 2003).

The iatrogenic and variant Creutzfeldt-Jakob diseases (iCJD and vCJD respectively) and kuru are infections forms of prion diseases associated with accidental transmission of PrP^{Sc} to human. Iatrogenic CJD is transmitted (Human-to-human transmission) by cadaveric dura mater grafts, stereotactic intracerebral EEG needles or neurosurgical instruments, intramuscular injections of contaminated cadaveric pituitary-derived human growth hormone (hGH) and gonadotrophin hormone. Most cases of iCJD are attributed to treatment with hGH (Haïk & Brandel 2014). Variant CJD was first described in 1996 and the earliest case had an onset of the illness in 1994 (Will et al. 1996). Until 2014, 229 vCJD cases have been diagnosed worldwide. It is believed that vCJD is due to BSE contamination of food. The epidemiological evidence strongly supports the view that the agent passed from cattle to human (Knight 1998). The phenotype of vCJD is the frequent psychiatric

symptoms (depression, anxiety, apathy, withdrawal and delusions) and atypical pain in limbs or face at clinical onset.

Kuru is found among people from New Guinea who practiced a form of cannibalism in which they ate the brains of dead relatives as part of a funeral ritual. The clinical hallmarks of this disease are shivering (original Fore translation of "Kuru") and severe cerebella ataxia accompanied with tremors (Hornabrook 1968).

1.1.3 Conversion of PrP^{C} into PrP^{Sc}

Conformational change of the cellular prion protein (PrP^{C}) to the prion protein scrapie (PrP^{Sc}) is considered the central event of the formation and pathogenesis of the infectious agent. In infection it is assumed that PrP^{Sc} comes into contact with PrP^{C}, and this contact induce the conformational transition of PrP^{C} to PrP^{Sc} (Figure 2). Chaperone actions describe induced conformational changes of proteins, hereupon PrP may act as its own chaperone (Rabenau et al. 2000). In contrast to the α-helical-rich structure of PrP^{C}, the tertiary structure of PrP^{Sc} shows a higher content of β-sheets (Riesner 2003). The misfolded PrP^{Sc} molecule seems to trigger formation of oligomeric structures, which then tend to form fibrils (Silveira et al. 2005; Walsh & Selkoe 2004). The newly generated PrP^{Sc} will be grouped in order to form new aggregates, or it will be integrated into already existing aggregates. These aggregates of PrP^{Sc} will be stabilized by aggregation thereby acquiring a much longer turnover time. The conformational change of PrP^{C} to PrP^{Sc} might go on as long as new PrP^{C} molecules are synthesized in the cell (Wadsworth et al. 2003; Knight et al. 2004).

1.1.4 Structure and proteolytic processing of the cellular prion protein

The cellular prion protein PrP^{C} is a membrane glycoprotein, it is attached to the lipid bilayer via C-terminal, glycosyl-phosphatidylinositol (GPI) anchor (Westergard et al. 2007). The matured PrP^{C} exhibits 208-220 amino-acid residues depending on species and the molecular mass vary between 36 to 27 KDa, depending on the extent of glycosylation. PrP^{C} consists of three glycosylated forms

(unglycosylated, monoglycosylated and diglycosylated) (Riesner 2003). The three-dimensional structure of PrP^C includes a disordered N-terminal domain (residues 23–124; numbering for mouse PrP) and a C-terminal globular region (residues 125–228) composed of three α-helices and two short β-strands (Figure 3) (Riek et al. 1997; Knaus et al. 2001). The N-terminal region of PrP^C encompasses a polybasic region (residues 23–27) and a series of histidine-containing octapeptide repeats (residues 51–90), which can bind metal ions such as Cu^{2+} (Knaus et al. 2001). The residues encompass a charged region followed by a highly conserved hydrophobic domain (residues 112-130), which acts as a transmembrane anchor (Hegde et al. 1998). The N-terminal signal peptide (residues 1-22) is removed and GPI anchor is attached at residue 230, during ER biosynthesis (Stöckel et al. 1998). Two N-linked oligosaccharide chains are also added (at Asn-180 and Asn-196) (Stahl et al. 1987). The cellular proteases cleave proteolytically some amino residues of the protein (near residue 111) to generate N- and C-terminal fragments called N1 and C1, respectively (Gorodinsky & Harris 1995; Vincent et al. 2001; Biasini et al. 2012).

The biosynthetic pathway of PrP^C is similar to other membrane and secreted proteins, involving synthesis on ER-attached ribosomes, transit to the Golgi, followed by delivery to the cell surface (Harris 1999).

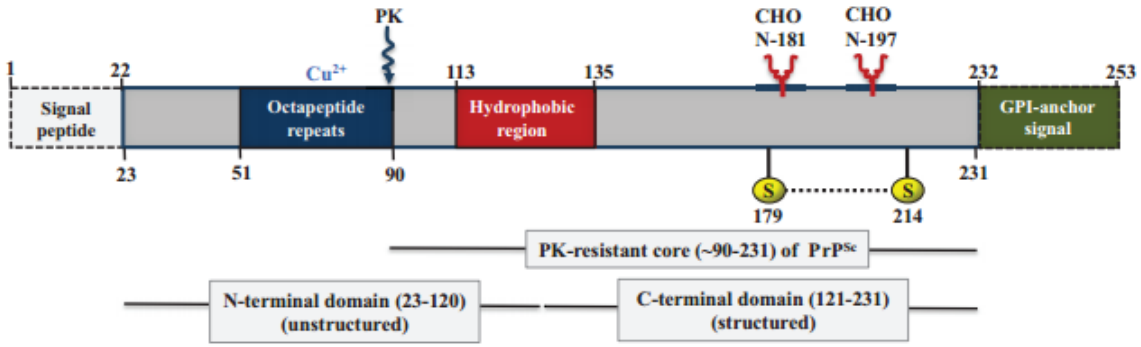

Figure 3. Organization of human PrP. The unprocessed PrP is 253 amino acid residues in length and includes a signal peptide (1–22), four OR, a hydrophobic region (113–135), one disulphide bond between cysteine residues (179–214), two N-linked glycosylation sites (at residues 181 and 197), and a GPI-anchor attached to the C-terminus of PrP replacing the GPI-anchor signal (residues 232 to 254). The four OR in the N-terminal domain have a high affinity for copper ions (Cu2+), while a preceding oligopeptide (PQGGGGWGQ) lacks the histidine that is necessary to bind a Cu2+ ion. Mutated forms of PrP can contain insertions of one to nine additional OR or a deletion of one OR. A palindromic region, AGAAAAGA (113–120), lies in the hydrophobic region (113–135) and is thought to

be important in the conversion of PrP^{C} to PrP^{Sc}. OR: Octapeptide repeat; GPI: glycophosphatidylinositol; PK: proteinase K; CHO: carbohydrates (Acevedo-Morantes & Wille 2014).

1.1.5 Cellular prion is ubiquitously expressed

PrP^{C} is expressed in all cell types although its expression is consistently higher in the nervous system cells (Martins et al. 2002; Martins et al. 2010; Linden, Martins, M. A. M. Prado, et al. 2008). The human PrP^{C} is encoded by the *PRNP* gene chromosome at 20p13 (*Prnp* in mouse) and consists of two exons (Basler et al. 1986). All known mammalian *Prnp* genes are encoded by a single open reading frame (ORF). The expression begins in early embryogenesis, and it is present at highest levels in neurons of the brain, in particular in synapses, and spinal cord in adults (Manson et al. 1992; Harris et al. 1993) and moderate levels in spleen, liver, heart and lung whereas kidney showed the lowest expression (Ning et al. 2005). The levels of prion protein is not related with PrP^{C} mRNA levels, this suggests that PrP^{C} is mainly post-translational regulated in CNS neurons (M J Ford et al. 2002).

1.1.6. Physiological function of PrP^{C}

Many recent studies aim to elucidate the physiological function of the PrP^{C}, however their function is not fully clarified. Although, a behavioral study reveals specific age-dependent differences between WT and $Prnp^{0/0}$ mice, which indicate an important role for PrP^{C} in brain function (Schmitz et al. 2014). Overexpression of PrP^{C} seems to be protective in cell lines and primary neurons from several kinds of apoptotic stimuli (Shyu et al. 2005; Milhavet & Lehmann 2002; Kim et al. 2004; Roucou et al. 2003; Diarra-Mehrpour 2004). Beyond that, PrP^{C} has been shown to play a role in regulating intracellular signaling cascades, including those mediating cellular survival (Lo et al. 2007). For example, in Knockout PrP^{C} (*Prnp*-null) animals circadian rhythm was altered (Tobler et al. 1996), and abnormal synaptic structure in the hippocampal formation was observed (Brown 2001). Several functions have been proposed for PrP^{C} including a role in oxidative stress (Das et al. 2010), immune modulation (Panigaj et al. 2011), differentiation (M. J. Ford et al. 2002), translocation of metals, such as copper (Pauly & Harris 1998), alteration of copper, zinc and iron

homeostasis in the brain (Pushie et al. 2011), cell adhesion; and transmembrane signaling (Linden, Martins, M. a M. Prado, et al. 2008). Most studies indicate that PrP^C may be associated with multi-molecular membrane complexes, which mediate several functions in distinct cellular compartments (Kim et al. 2004). The availability of PrP^C at the cell surface depends on its cycle between plasma membrane and endocytic compartments. This process is also involved in pathways of internalization that may be critical for PrP^C physiological functions (Negro et al. 2001). Several studies have indicated a direct high affinity interaction between Aβ (neurotoxic peptide in AD) and PrP^C. Indeed, there is evidence that PrP^C may play a critical role in the pathogenesis of Alzheimer's disease (Kellett & Hooper 2009).

1.2 Alzheimer's disease

Alzheimer's disease (AD) is the most prevalent age-related neurodegenerative disease (around 60% of dementia cases) that leads to cognitive impairment and dementia (Rial et al. 2012). The principal risk factor for AD is age, but it is not a normal part of aging. Approximately 5-10% of population exhibiting an age of 65 years have AD and the prevalence of this disease increases with increasing age from 19% to 30% after 75 years of age (Figure 4) (Iris Medical Education 2015).

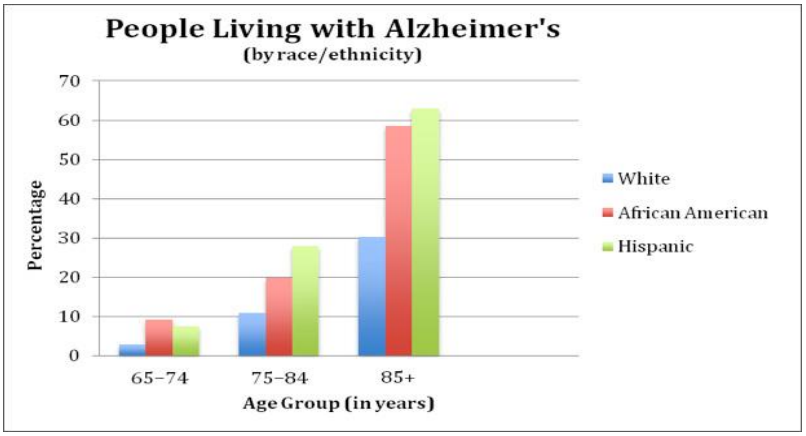

Figure 4. Percentage of people with Alzheimer's disease worldwide (Iris Medical Education 2015).

It has been identified two distinct forms of AD, the familial Alzheimer's disease (FAD) and the sporadic Alzheimer's disease (SAD) (Ling et al. 2003; Bertram et al. 2010). About 90-95% of Alzheimer`s disease represents a sporadic form and 5-10% represents familial form. AD is an irreversible, progressive neurodegenerative

disorder (Khachaturian & Radebough 1996). It leads to the progressive loss of mental, behavioral, functional decline and ability to learn (Anand et al. 2014). The definitive diagnosis is established post mortem through the histopathological analyses of patient's brain and there is none available effective treatment or preventive therapy (Eva Babusikova 2011). Neurochemical and pathological characteristic changes of AD are tissue atrophy caused by loss of synapses which is most striking in frontal and temporal areas of the brain cortex, gliosis and formation of two main protein clusters in extracellular and intracellular region of the brain (Eva Babusikova 2011). Extracellular deposits (amyloid plaques) occur most frequently in neocortex. They consist of 4kDa, 40-42 amino acid polypeptide chain called amyloid β peptide (Aβ) (Glenner & Wong 1984). The intracellular deposits are generated from filaments of microtubular hyperphosphorylated tau protein leading to the neurofibrilar tangles (Alonso et al. 2008; Grundke-Iqbal et al. 1986; Lee et al. 1991). In AD the dysregulation of intracellular calcium plays an important role in the pathogenesis. It is thought that neurodegeneration induced by Aβ and hyperphosphorylated tau may be mediated by changes in calcium homeostasis. Persistent changes in calcium homeostasis are proximal reasons of neurodegeneration in AD patients (Khachaturian 1989). Tau is a neuronal microtubular associated protein and it is assumed that it plays a major role in the conservation of cells shape and in axonal transportation (Buée et al. 2000). Amyloid plaques and neurofibrilar tangles are characteristic of AD, and they are present in different neurodegenerative pathological situations (Robert & Mathuranath 2007). The aggregates are involved in a process which leads to progressive degeneration and to neuronal death. Two main hypotheses explaining the cause of AD development were proposed: (1) hypothesis of amyloid cascade – a neurodegenerative process is a serial of events started by an abnormal processing of amyloid precursor protein (APP) [Hardy and Higgins, 1992], and (2) hypothesis of neuronal cytoskeletal degeneration (Braak & Braak 1991). According to the amyloid hypothesis accumulation of amyloid beta (Aβ) plaques acts as a pathological trigger for a cascade that includes neuritic injury, formation of neurofibrillary tangles via tau protein hyperphosphorylation, leading to neuronal dysfunction and cell death in AD (Hardy & Higgins 1992; Selkoe 1999; Dickson

1997). The cellular mechanisms of Aβ oligomer/plaque formation neurotoxicity are, however, controversial (Rushworth et al. 2013).

1.2.1 Familial Alzheimer's disease (FAD)

In humans, FAD is caused by autosomal dominant mutations in three genes. Gene for amyloid precursor protein (APP) 21q21.3 (Goate et al. 1991), presenilin 1 (*PSEN1*) on chromosome 14q24.2 and presenilin 2 (*PSEN2)* on chromosome 1q42.13 (Campion et al. 1995; Cruts et al. 1995; Sherrington et al. 1996). These mutations are responsible for a high production of Aβ that may accumulate and form amyloid plaques. The gamma-secretase complex include presenilins and wich are involved in amyloid processing of APP. PSEN1 and PSEN2 mutations are linked to early onset of AD. Mutations of presenilin 1 may cause the cleavage alterations of APP and production of $A\beta_{1\text{-}42,}$ the most pathological variant for generation of amyloid plaques in the brain (Xia et al. 1997; Eva Babusikova 2011). Aβ level increase years before any clinical symptoms of Alzheimer's disease are observed. Interestingly, mutations in the tau gene are not associated with AD.

1.2.2 Sporadic form of Alzheimer's disease

The sporadic for of AD is thought to be a multifactorial combination of aging, genetic predisposition, and exposure to environmental agents including low education, head trauma, toxins and viruses. However, no environment agents have been proven to be directly involved in pathogenesis of AD (Bird 2015). Apolipoprotein (apo) E4 has been genetically linked to late-onset of AD. An individually carrying a mutation on APOE4 allele have 3 to 15 times increased risk of developing AD. Risk genes increase the likelihood of developing a disease, but do not guarantee it will happen (Duthey 2013).

1.2.3 Physiological function and processing of Amyloid precursor protein

The amyloid precursor protein (APP) is a type I transmembrane glycoprotein that is expressed in a wide variety of mammalian cells (Müller-Hill & Beyreuther

1989). The physiological function of APP and its homologues remains unclear, but its role has been suggested in neural protein trafficking along the axon, cell adhesion, neurite outgrowth and synaptogenesis and calcium metabolism (Zheng & Koo 2006).

In humans, of the 19 exons which encode APP, exons 7, 8, and 15 are subject to alternative splicing (Ling et al. 2003). APP variants result from differential splicing of APP during transcription. The common expressed isoforms of APP have 770, 751 and 695 amino acid residues. APP695 is the predominant neuronal isoform (Kang et al. 1987).

The mRNA splice variants of APP are expressed in different cells in different amounts. Since APP has a widespread expression and distribution it has been suggested to play an important role (Dawkins & Small 2014). APP can be post-translationally processed by enzymes termed α-, β-, and γ-secretases, which can cleave the protein to produce and release a number of smaller amyloid fragments (Selkoe 2001; De Strooper & Annaert 2000). On the cell surface, the APP can be cleaved by α-secretase (non-amyloidogenic pathway) or β-secretase (amyloidogenic pathway) (Figure 5). The non-amyloidogenic processing of APP occurs via the α-secretase pathway, which cleaves on the C-terminal side of residue 16 of the Aβ sequence, generating a 83-residue C-terminal fragment (C83) and releases a large soluble ectodomain of APP called sAPPα (Esch et al. 1990). Subsequent cleavage by γ-secretase releases a short peptide (p3) and generates the APP intracellular domain (AICD). In the amyloidogenic pathway the β-secretase cleaves APP between Lys16 and Leu17 or Tyr10 and Glu11 to produce sAPP-β that is released to the extracellular medium, the fragment that stayed embedded in the membrane is called C99. This fragment (C99) is subsequently cleaved by y-secretase in a cleavage to produce Aβ peptide and the smaller AICD (Pitsi & Octave 2004; Maltese et al. 2001; Motoki et al. 2012; Vetrivel et al. 2009). The majority of Aβ have 40 amino acids residues in length ($A\beta_{1\text{-}40}$), however the γ-secretase cut is imprecise and creates Aβ isoforms of different lengths, between 39 and 43 amino acids residues in length. The length of Aβ peptide varies at C-terminal according to the cleavage pattern of APP. The Aβ1–40 isoform is the most prevalent, followed by Aβ1–42 which is hydrophobic in nature and aggregates at a faster rate than Aβ1–40 (Walsh & Selkoe 2007; Perl

2010). Aβ1–42 is the major protein component of amyloid plaques in the Alzheimer's disease brain (Masters et al. 1985).

1.2.4 β-amyloid peptide (Aβ)

In AD, amyloid fibrils are formed from Aβ peptide (Finder & Glockshuber 2007). This peptide is produced at cholesterol-rich regions of neuronal membranes and secreted into the extracellular space (Simons et al. 1998). The cleavage of APP, in amyloidogenic pathway, results in a number of Aβ isoforms, Aβ1-40 and Aβ1-42 are the most commonly found. These two forms are capable to adopt many differently shaped aggregates including amyloid fibrils (Meinhardt et al. 2009; Lührs et al. 2005; Kodali & Wetzel 2007; Sachse et al. 2008) as well as nonfibrillar aggregates that are sometimes also termed Aβ “oligomers” (Haass & Selkoe 2007; Glabe 2008). The more amyloidogenic isoform is Aβ1-42. It aggregates more readily and it is predominantly found in senile plaques. Despite Aβ peptide and Aβ amyloid plaques are commonly outside the cell (Pepys 2006; Meyer-Luehmann et al. 2003), considerable evidences points towards a potential relevance of intracellular Aβ (Takahashi et al. 2002; Rajendran et al. 2007; Oakley et al. 2006; Du et al. 2008). Monomeric Aβ is generally non-toxic, although there is evidence that Aβ oligomers are responsible for the synaptic dysfunction that occurs in AD (Walsh et al. 2002; Cleary et al. 2005). Aβ oligomers are toxic for cells (Yankner et al. 1989) by different pathways and their toxicity correlates with the level of aggregation. The peptide is capable to influence metabolic pathways in the brain. It is able to activate effectors of apoptosis, caspases, to affect calcium homeostasis by increasing the amount of intracellular calcium concentration (Mattson et al. 1993; Eva Babusikova 2011), and to induce neuronal death.

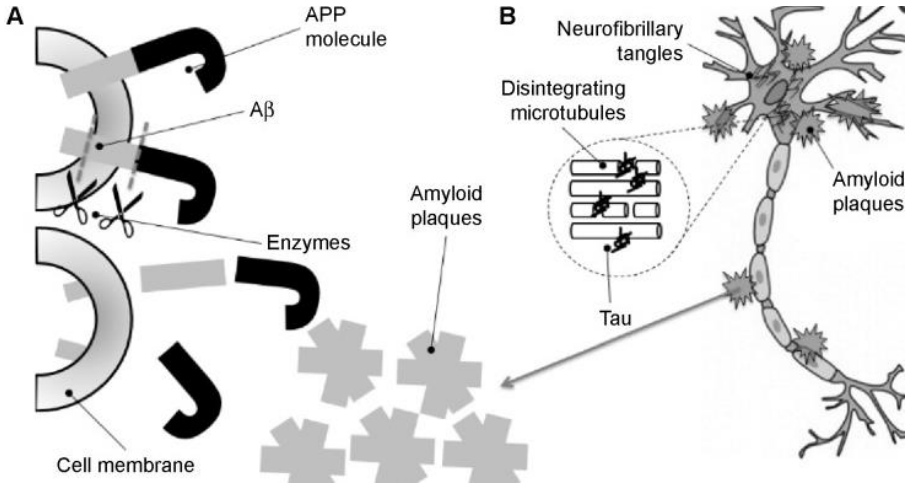

Figure 5. Formation of amyloid (**A**) and neurofibrillary tangles (B) in the neurons in Alzheimer's disease (Fonseca-Santos et al. 2015).

2. **Prion protein and Alzheimer's disease**

There are several pathological similarities and genetic connections among AD and prion diseases. The coexistence of AD in CJD has been described (Hainfellner et al. 1998), and PrP^C has been shown to co-localize with Aβ plaques (Voigtländer et al. 2001). The PrP^C- Aβ plaques were present in most of CJD patients with AD-type pathology (Del Bo, *et al.*, 2006) and it has been postulated that PrP^C may promote Aβ plaque formation, also a genetic correlation among PrP^C and AD has been reported. However, there was no evidence of an interaction between both proteins involved in these diseases despite pathological and genetic links between them (Schwarze-Eicker *et al.*, 2005).

3. Aims of the present study

The main task of the present study is to investigate the role of the cellular prion protein (PrP^C) in AD, which is still unclear because previous studies showed contradictious results (Parkin et al. 2007; Laurén et al. 2009). While Parkin *et al*. (2007) observed an inhibitory function of PrP^C on the processing of APP resulting in a reduced amount of Aβ, Laurén *et al.* (2009) reported an interaction of PrP^C and Aβ which enhances the toxicity of Aβ. By this, PrP potentially acts as receptor for amyloid beta mediating the toxicity of amyloid beta-oligomers in an AD mice model (Laurén et al. 2009; Gimbel et al. 2010). To elucidate this issue, the main objective of the present study is to investigate the influence of a knockout of the *Prnp* gene in 5XFAD mice, exhibiting 5 mutations related to familial Alzheimer disease. These mice express the 695 aminoacids isoform of the human amyloid precursor protein (APP695) carrying the Swedish/London/Florida mutations under the control of the murine Thy-1-promoter. In addition, human presenilin-1 carrying the M146L/L286V mutations is expressed also under the control of the murine Thy-1-promoter. These mice are commercially available and show an $A\beta_{1\text{-}42}$ accumulation and an increased neuronal loss during aging (Eimer & Vassar 2013). To create a bi-transgenic mice line 5xFAD mice were crossed with $Prnp^{0/0}$ Zurich 1 mice (Büeler et al. 1992).

In the present study, the role of PrP^C in AD shall be defined by subjecting two transgenic mice (5xFAD and $Prnp^{0/0}$5xFAD) at different ages (3, 9, 12[1] months of age) to a battery of behavioral task, including the open field, elevated plus maze, novel object recognition rotarod task. With these tests, potential deficits in mice behavior (locomotor activity, learning and memory performance, motor function or anxiety behavior) will be analyzed in 5xFAD and $Prnp^{0/0}$5xFAD mice in comparison to a corresponding control group consisting of PrP^C wild type (WT) and PrP^C knockout mice ($Prnp^{0/0}$). In addition, comprehensive biochemical analysis, which include western blotting, ELISA, immunohistochemical staining should be undertaken to quantify the amount of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in mice during aging, to analyze the amount and distribution of Aβ deposits dependent of PrP, and to investigate the regulation and potential involvement of downstream signaling proteins in the Aβ induced toxicity dependent of the PrP concentration.

[1] 12 months of age group is only composed by $Prnp^{0/0}$ and $Prnp^{0/0}$ 5xFAD due to the low availability of 5xFAD. 5xFAD mice have a high mortality after 9 months of age.

II. Materials

1. Chemicals

All chemicals used in this study were obtained from Applichem (Darmstadt, Germany), Biochrom AG (Berlin, Germany), Bio-Rad (München, Germany) Merck (Darmstadt, Germany) Roth (Karlsruhe, Germany) and Sigma-Aldrich (Steinheim, Germany) if not stated otherwise in the text.

Reaction tubes, pipette tips and laboratory glassware were autoclaved or sterile prior to use.

1.1 Antibodies

Antibodies used for immunoblotting (IB) and immunohistochemistry (IHC) are listed in Table 2.

Table 2. List of antibodies and respective dilutions were used for Western blot analysis after SDS-PAGE.

Antibody	Dilution	Supplier
APP	1:1000	Abcam, Cambridge, UK
Anti-β-actin-antibody	1:1000	Abcam, Cambridge, UK
Anti-Fyn	1:1000	Abcam, Cambridge, UK
Anti-P-Fyn	1:1000	Abcam, Cambridge, UK
Anti-Caveolin 1	1:1000	Abcam, Cambridge, UK
Anti-GAPDH	1:1000	Abcam, Cambridge, UK
Anti-SAF-32	1:1000	Abcam, Cambridge, UK
Anti-beta Amyloid	1:500	Abcam, Cambridge, UK
AP-conjugated anti-mouse IgG	1:2000	Santa Cruz Biotechnology, Santa Cruz, USA
AP-conjugated anti-rabbit IgG	1:2000	Santa Cruz Biotechnology, Santa Cruz, USA

1.2. Kits

All the listed kits were used according to the manufacturer's instructions.

Table 3. List of the kits used in this study

Name	**Company**
DNeasy Blood & Tissue kit	Qiagen, Hilden, Germany
Elisa essay Aβ1-40	IBL, International
Elisa essay Aβ1-42	IBL, International

1.3. Oligonucleotids

All mice were genotyped by PCR amplification for 5xFAD and $Prnp^{0/0}$ mutation and $Prnp^{+/+}$.

Table 4. List of Oligonucleotids

Primer	**Sequence in 5' – 3' orientation**	**Supplier**
Mu_PrP_WT_for ($Prnp^{+/+}$allele)	ATGGCGAACCTTGGCTACTGGGCTG	Operon
Mu_PrP_WT_Rev ($Prnp^{+/+}$allele)	CATCCCACGATCAGGAAGATG	Operon
oIMR3611_For 5xFAD	CGGGGGTCTAGTTCTGCA	Operon
oIMR3610_Rev 5xFAD	AGGACTGACCACTCGACCAG	Operon
P3 ($Prnp^{0/0}$ allele)	ATTCGCAGCGCATCGCCTTCTATCGCC	Operon
P4 ($Prnp^{0/0}$ allele)	CATCCCACGATCAGGAAGATG	Operon

1.4. DNA and Protein Standards

All the listed DNA and Proteins Standards were used according to the manufacturer's instructions.

Table 5. List of DNA and Protein Standards

DNA and Protein Standards	Supplier
100bp Standard DNA marker	New England Biolabs, Frankfurt am Main, Germany
Precision Plus Protein Standards (dual color)	Bio-Rad, München, Germany

2. Buffers and Solutions

Blocking solution for WB: 5% milk powder in TBST

Electrophoresis buffer (SDS-running buffer): 192mM Glycine, 0.1 SDS, 25mM Tris-HCL pH 8.3.

ECL (A): 2.5mM Luminol, 0.4mM p-Coumar acid, 0.1M Tris-HCL pH 8.5

ECL (B): 18% H_2O_2, 0.1M Tris pH 8.5

Lysis buffer (tissue): 50mM Tris pH 8.0, 150mM NaCl, 1% Triton-X-100

TBST (10x): 200 mM Tris, 1.5M NaCl, 1% Tween-20

Transfer Buffer for WB: 192mM Glycine, 20% Methanol, 25mM Tris-HCl pH 8.3

3. Instruments and other materials

Table 6- List of the instruments used in this study

Appliance	Model or Description	Manufacture
Bio-safety Cabinet	Hera safe KS	Heraeus/ Osterode, Germany
Centrifuge	5415C	Eppendorf/Hamburg,Germany
Centrifuge	Rotina 35R	Hettich/ Tuttlingen, Germany
Chamber slide	Lab-Tek™ II Chamber Slide, 15453	nunc/ New York, USA
Electro blotting apparatus	Mini Trans-Blot®	Bio-Rad /Munich, Germany
Electrophoresis apparatus	Mini-Protean® III	Bio-Rad /Munich, Germany
Heated magnetic stirrer	iKAMAG RCT	IKA-Labortechnik/Staufen, Germany
Ice machine	-	Ziegra /Isernhagen, Germany
Microscope	Zeiss LSM 510 Meta	Carl Zeiss/ Goettingen, Germany
Power supply	Power Pac 300	Bio-Rad /Munich, Germany
Safe-Lock tubes	0.2, 0.5, 1.5 and 2ml	Eppendorf /Hamburg, Germany
Serological pipettes	2, 5, 10, 25ml	Sarstedt /Germany
plastic tubes	15 and 50ml	Sarstedt /Germany
pH meter	pH 526	WTW*I* Weilheim, Germany
Shakers	CERTOMAT R	Sartorius/ Goettingen, Germany
Spectrophotometers	EL808	Bioteck instruments/Winooski-vermont, USA
Sterile filter Nalgene	0.2µm	Sartorius/ Goettingen, Germany
Sterile filter pipette tips	-	Biozym /Oldendorf, Germany
Thermomixer	5436	Eppendorf/ Hamburg, German
Vortexer	Genie 2™	Bender and Hobein /Zurich, Switzerland

4. Software

The following is a list of scientific software used in the study.

Table 7- List of scientific software

Program	Use	References
Graphpad Prism 5	Statistical analysis	GraphPad Software, Inc. California, USA
ImageJ 1.43u	Densitomatric analysis	National institutes of Health, USA
KC4 V3.4	Absorbance reader	Bioteck instruments, USA
LabImage 2.7.1	Densitomatric analysis	Kapelan GmbH, Halle, Germany
Zeiss LSM 4.2.0.121	Immunofluorescence	MicroImaging GmbH, Goettingen, Germany

III. Methods

1. Molecular experiments

1.1. Extraction of genomic DNA

The DNA was purified from tail biopsies with DNeasy Blood & Tissue kit (Qiagen). The piece of the tail (0.4cm) was placed in a microcentrifuge tube. It was added 180 µl Buffer ATL and 20µl proteinase K. After addition the sample was briefly mixed by vortexing and incubated at 56ºC overnight. The samples were mixed by vortexing after lysis. Following addition of AL solution and 100% ethanol and mixed. The mixture (including any precipitate) was pipetted DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min. The mini spin column was placed in a new collection tube and 500 µl Buffer AW1 was added and centrifuged for 1 min again. The mini spin column was replaced for a new one and added 500 µl Buffer AW2, and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane. The DNeasy Mini spin column was placed in a clean 2 ml microcentrifuge tube, and pipetted 200 µl Buffer AE directly onto the DNeasy membrane. It was Incubated at room temperature for 1 min, and then centrifuged for 1 min at 8000 rpm to elute. The last step was repeated twice.

1.2. Polymerase chain reaction

The choice of the target DNA is, of course, dictated by the specific experiment. However, one thing is common to all substrate DNAs and that is they must be as clean as possible and uncontaminated with other DNAs. When setting up multiple PCR experiments, it is better to assemble a mixture common to all reactions (master mix). Master mix was added to DNA. The experiment was performed in ice. Afterward all tubes were gently mixed and centrifuged to collect all liquid to the bottom of the tube. All mice were genotyped by PCR for 5xFAD and $Prnp^{0/0}$ mutation.

Table 8 – PCR Solutions

Component	**25 µl reaction**	**Final Concentration**
10X Standard *Taq* Reaction Buffer	2.5 µl	1X
10 mM dNTPs	0.5 µl	200 µM
10 µM Forward Primer	0.5 µl	0.2 µM (0.05–1 µM)
10 µM Reverse Primer	0.5 µl	0.2 µM (0.05–1 µM)
Template DNA	1 µl	<1,000 ng
Taq DNA Polymerase	0.125 µl	1.25 units/50 µl PCR
Nuclease-free water	to 25 µl	to 50 µl

1.2.1. **Thermocycling conditions for PCR**

For the PCR reaction the following programs were used:

Table 9 – PCR programs

Mutation	***5xFAD***		***Prnp $^{+}/^{+}$***		***Prnp $^{0}/^{0}$***	
STEP	**TEMP**	**TIME**	**TEMP**	**TIME**	**TEMP**	**TIME**
Initial Denaturation	95°C	3 min	95°C	3 min	95°C	1 min
30 C	95°C 56°C 72°C	30 seconds 60 seconds 1 minute	95°C 56°C 72°C	30 seconds 45 seconds 1 minute and 15 seconds	95°C 62°C 72°C	30 seconds 2 minutes 1 minute
Final Extension	72°C	6 minutes	72°C	10 minutes	72°C	5 minutes
Hold	4°C		4°C		4°C	

1.3. Preparation of mice's brain samples

The mice were sacrificed after behavioral experiment by euthanizing via CO_2 asphyxiation and then cervically dislocated. Dissection was carried out of neuronal tissue and brains separated bilaterally into two equal parts. The cortex that covers

the hippocampus was removed and used as sample for molecular experiments. The brains' parts and the cortex were stored at -80 ^{o}C.

1.4. Tissue Lysate

Immediately after mice sacrifice, brain tissues were frozen in dry ice and stored at -80 °C. The homogenization consists in placing a metallic bead in the eppendorf with frozen cortex sample and homogenize with ultra sonicator on ice. The tissue was lysed in lysis buffer (NaCl, Tris HCl, EDTA and Tx) containing protease and phosphatase inhibitor cocktail (Roche), for 30 min. Lysed tissue was centrifuged at 13000 rpm for 15 mins at 4 °C and supernatant was obtained for further protocols.

1.5. Bradford assay

The protein concentration was determined by the Bradford assay (Bio-Rad). Working solution was prepared by diluting a dye reagent concentrate (Bio-Rad) with dH20 in ratio 1:5 followed by filtration through whatmann filter paper. The protein standards were prepared in a concentration range between 0.05-1 mg/ml. Protein samples of unknown concentration were diluted 1:20 in dH2O. 980 µl of Bradford solution was added and samples were incubated for 10 min at RT. The absorbance of the samples was measured at 595 nm.

1.6. SDS-PAGE and Western blot

Western blotting uses specific antibodies to identify proteins that have been separated based on size by gel electrophoresis. The immunoassay uses a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane and application of an electrical current induces the proteins to migrate from the gel to the membrane. The membrane can then be further processed with antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents.

30 µg of normalized protein samples for protein content were adjusted to an equal final volume and mixed with 4x SDS loading buffer (Roti®-Load). After boiling at 95° C for 5 min samples were loaded into the SDS-polyacrylamide gel, 10–17 %. Proteins were separated in the gel, running at 100 V for approximately 2h and then transferred to a PVDF membrane (Amersham Bioscience) by blotting. After protein transfer the membrane was blocked at RT for 1h. The blocking solution was set in accordance with specific antibody requirements. First antibody was incubated at 4° C overnight. Subsequently the membrane was washed with TBST at RT 3 times for 5 min. Then the second antibody was applied at RT for 1h. Finally, after the final washing, the enzymatic reaction was performed using ECL solution and visualized with Molecular Imager ChemiDoc XRS+ with Image Lab Software (Bio-Rad). Densitometric values for each band intensity were determined using lab image 2.7.1 data analyzer software.

1.7. Enzyme Linked Immuno-Sorbent Assay (ELISA)

For ELISA analysis all procedures were performed according to supplier's recommendations. Briefly, equal amounts of protein from tissue lysates were added to a microtitre plate coated with a monoclonal antibody. The antigen binds to the immobilized (captured) antibody. After washing, a monoclonal antibody specific for the protein is added to the wells. Bound antibody is detected by the use of a secondary peroxidase-labeled antibody. After removal of excess of antibody, a substrate solution is added, which is activated by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of protein present in the original sample

1.8. Immunohistochemistry

Tissue slices from liver were made with cryosect, fixed with acetone. Immunohistochemistry was performed with following steps; The slides were blocked 10 minutes in 0.3% H2O2 solution in PBS at room temperature to endogenous peroxidase activity. After washing 3 times (PBS) the slides were blocked wit 10% fetal bovine serum in PBS. It was applied 6E10 antibody diluted in 1:500 an appropriately diluted primary antibody to the sections on the slides and incubated in a

humidified chamber for 90 min at room temperature. After 3 times washing with PBS, tissue slices were incubated with an appropriately diluted (1:200) biotinylated secondary antibody at room temperature 60 minutes. Incubation with secondary antibodies was followed by 3X washings with PBS. Extravidin (1:000) was placed for 30 minutes on slides. After washing step the slides were incubated with AEC solution (3-Amino-9-Ethylcarbazole) for 30 minutes and then washed with ddH_2O. The slides were immersed in hematoxylin for 13 seconds and rinsed in running tap water for 10 minutes. The slides were mounted with mounting medium (fluoromount) and covered with coverslips followed by microscopic examination.

2. Behavior experiments

2.1. Experimental Animals

Experiments were carried out with four different transgenic mice, wild type mice, 5xFAD mice (over expressed the K670N/M671L (Swedish), I716V (Florida), and V717I (London) mutations in human APP (695) and M146L and L286V mutations in PS1), PrP knockout ($Prnp^{0/0}$) mice (described by Bueler et al., 1992) and (bi)-transgenic mice line exhibiting $Prnp^{0/0}$5xFAD.

2.2. General procedures

The mice were housed one per cage in a temperature-controlled room (23±2°C) with 12-h light/dark cycle. All behavioral testing were performed during the light portion of the cycle. Mice were 3–14 months old and they had free access to food and water in their home cages.

Experimental mice were carried to the test room in their individual cages, 30 min prior to test beginning for adaptation to the condition of the behavior testing room.

2.3. Nest Building

Behaviors associated with nest building are highly linked to survival capacity of wild mice. The nest provides a shelter from predators, hiding-place or camouflaging the mouse. The small size of mice make them vulnerable to heat loss and the nest protect them against harsh environments. Male and female mice build a nest when they are provided with a suitable building material. They are highly motivated to build a nest even in captivity. The changes in the highly motivated behaviors, or the nest itself, indicate a significant alteration in the environment or of animals behaviour, for instance, pain, illness, or other stressors that may affect or reduce animal's overall welfare (Gaskill et al. 2013).

On the first day of testing, one piece of a tissue paper (36 cm x 12 cm) was placed in the cage to facilitate nest building. The presence and the quality of the nest were evaluated on the following day on a modified five-point scale according to the method of Deacon (Deacon 2006).

A) Tissue not noticeably touched (>90% intact, 1 point)

B) Tissue partially shredded (50-90% remained intact, 2 points)

C) Mostly shredded but not identifiable as a nest (>50% of the tissue is shredded, 3 points).

D) A nearly intact nest can be identified, with flat walls (>90% of the tissue is shredded, 4 points)

E) An intact nest, with a crater whose walls are higher than mouse body height (100% of the tissue is shredded, 5 points).

2.4. The open field

The open field (OF) test provides an opportunity to systematically assess novel environment exploration, provides an initial screen for anxiety behavior and measure general locomotor activity levels into the arena in rodents (Bailey & Crawley 2009). The test is based on conflicting innate tendencies to avoid the bright light and open spaces (that ethologically mimic a situation of predator risk) and of exploring novel environment. (*Open-field test for anxiety*) The number of line crosses and the

frequency of rearing are usually used as measures of locomotor activity, exploration and anxiety. The number of central square entries and the duration of time spent in the central square are measures of exploratory behavior and anxiety (*MPD - Brown1 - protocol*).

The experimental animals were placed in a dimly observation cage (72x72cm). The maze was divided virtually in sixteen 18x18cm squares (squares number six, seven, ten and eleven correspond to the imaginary central zone and the remaining twelve squares correspond to peripheral zone). The activity of the mice was monitored by a video motility system (Video-Mot II, TSE, Bad Homburg, Germany). Locomotor activity (squares crossed) and anxiety (locomotion in center squares) was measured. The open field was used in a single testing session. The mice were placed in one of the four corners of the open field and were allowed to explore the apparatus for 5 minutes. After the test, the apparatus was cleaned with 70% ethanol and allowed to dry between tests.

2.5. Novel Object Recognition

Novel object recognition is a test of learning and memory. The NOR test becomes commonly used for investigation of memory alterations. The mice were exposed to specific objects in a single trial. During the test trial, one familiar object was removed and replaced by a novel object. The preference for a novel object means that the familiar object exists in animals' memory (Ennaceur 2010). The total time spent exploring both objects during the test and training phase and discrimination index, i.e., the difference between the time spent exploring novel object and familiar object, during test phase can be considered (Antunes & Biala 2012).

The experimental apparatus consisted of a white rectangular open field (72x72cm). For performing the test we used two identical objects and other different from these two. The objects were a plastic rectangular box and a lego piece. The activity of the mice was monitored by a video motility system (Video-Mot II, TSE, Bad Homburg, Germany). A 3-day procedure was used. Habituation took place by exposing the animal to the experimental apparatus for 5 min in the absence of

objects one time. Twenty four hours after the habituation, two identical objects were placed in the open field. The mice were placed in an opposite corner to the objects into open field maze and they were allowed to explore for 5 minutes. After a retention interval of 24h, mice were placed again in the apparatus with the same two identical objects for 5min. Post-familiarization session the animals were transferred to their home cages for 5min interval. Following this period, the animal was re-introduced into the arena for the test session. Now, a triplicate of the familiar and a novel object were placed in the arena and the animal was allowed to explore during a 5 minutes test session. The open field and the objects were cleaned with 70% ethanol to minimize the olfactory cues between mice.

The percentage of time spent exploring the novel object (TN) relative to the total time spent exploring both objects (TN+TF) was used to calculate the index of recognition memory [RI = TN/(TN + TF)].

2.6. Elevated Plus Maze

The elevated plus maze is a simple method for assessing anxiety responses of rodents (described (Pellow et al. 1985). It is given the choice to the mice of spending time in open, unprotected maze arms or enclosed, protected arms, all elevated approximately 50cm from the floor. The test relies upon rodents' proclivity toward dark, enclosed spaces (approach) and an unconditional fear of heights/open spaces (avoidance) (Brain 1976). This approach–avoidance conflict results in behaviors that have been correlated with increases in physiological stress indicators (Holmes et al. 2003). The relevant behaviors are the time spent and entries made on the open and closed arms.

The apparatus used for the elevated plus maze test comprises two open arms 60cm across from each other and perpendicular to two closed arms 60cm with a center platform 10cmx10cm. The closed arms have a high wall 20cm to enclose the arms, whereas the open arms have no walls. The entire apparatus is 50cm above the floor and is placed in an empty square (70x70cm) to protect the fallen mice to escape during the experiment.

The mice were placed in the center of the platform and they were allowed to explore the apparatus for 5 minutes, during this time a video tracking system recorded their behavior (Video-Mot II, TSE, Bad Hamburg, Germany). After the test, the apparatus was cleaned with 70% ethanol and allowed to dry between sessions. The time spent in the open arms, closed arms and central zone was analyzed.

2.7. Rotarod

Before aspects of behavior such as emotionality or cognition it is vital to determine motor capabilities of mice. The rotarod is designed to test balance and motor coordination in which the animal is placed on a horizontal rod that rotates about its long axis. The animal must walk forward to remain upright and not fall off (Deacon 2013). This test requires motor skills, including coordination of the body. It is measured the time (latency) it takes the mouse to fall off the rod rotating at different speeds or under continuous acceleration (e.g. from 4.0 to 40rpm).

To measure motor coordination mice were placed on a computerized treadmill (TSE rotarod system). Mice were trained for 5 consecutive days at constant speed (5.0 rpm) for a maximum duration of 280 sec. per trial. The testing day consisted of 2 trials with 5min intertrial intervals, mice were placed on an accelerating rotarod from 4.0 – 40rpm for 280 sec and the latency to fall was measured. Rotarod performance was assessed by evaluating the best trial out of two performed in the test day.

2.8. Fear Conditioning

Fear conditioning is a test of memory and learning in which mouse learn to associate a context and cue with an unconditioned stimulus. During training phase, the mouse was exposed to a chamber in which the cue (white noise) is paired the stimulus (footshock). When the animal is returned to the same environment without the footshock and cue, it generally will demonstrate a freezing response if it remembers and associates that environment with the aversive stimulus. On a second trial it is presented to the mouse an altered context and is introduced the white noise

cue, once again, if the mouse remembers the aversive stimulus it will display a freezing behavior, which is the normal response of fearful situations. Freezing behavior is defined as complete immobility with the exception of breathing. The freezing episodes and time are recorded for evaluation (Shoji et al. 2014).

The apparatus for the fear conditioning and context test is an acrylic square chamber (31.8 cm x 25.4 cm x 26.7 cm) with a electrifiable metal grids floor, a sound source, calibrated shock generator and video system (VFC system, Med Associates).

The conditioning step consists in placing the mice in the chamber and allow them to explore the chamber for 180 seconds with a background sound. After that, a tone is presented as a conditioned stimulus, and a 0.5 mA footshock is given to the mice as an unconditioned stimulus during the last 2 seconds of the sound. The mice remained in the chamber for 30 seconds after the footshock.

Twenty-four hours after the conditioning session, the mice are returned to the same conditioning chamber for 180 seconds for the contextual test in absence of the tone and the footshock. The freezing behavior (absence of motion excluding the respiration) was measured by video system (VFC system, Med Associates). The cued test was performed fallowing the context test. In this test, the mice were placed into a different context, smooth white floor that covered the metal grids and a curved white wall that covered the wall of the chamber. This provides a new context that is unrelated to the conditioning chamber. They were allowed to explore the chamber during 210 seconds and in the last 180 seconds was presented the auditory cue that at the time of conditioning was given to the mice. The freezing time was measured by video system (VFC system, Med Associates). The chambers were cleaned between mice in all tests.

IV. Results

The present study analyses the function of PrP^C in a mouse model with 5 familial AD mutations. For this a novel bi-transgenic mice, $Prnp^{0/0}$5xFAD, exhibiting a gene knock out of *Prnp* (PrP^C gene) was created. Different mice were stratified into four groups: 1) 5xFAD, 2) WT, 3) $Prnp^{0/0}$5xFAD and 4) $Prnp^{0/0}$. Groups 1 and 2 expressed the PrP^C wild type gene on both alleles. To focus only on the Aβ induced toxicity in mice behaviour and not on a potential effect of a *Prnp* knockout, the group 1 was compared with group 2 as well as group 3 was compared with group 4.

A second aim is to investigate the Aβ induced toxicity during aging. For this reason we created three different aging groups: A young 3 month old group, a middle aged 9 month old group and an old age >12 months of age.

1. Behavioural results

1.1 Increase of life span in $Prnp^{0/0}$5xFAD mice

To investigate the impact of PrP^C on the life span of 5xFAD mice, the mice were housed under controlled temperature conditions (21-22°C) and had free access to food and water in a 12:12 h light: dark cycle. During a period of two years the survival of 5xFAD mice was monitored. Disease animals were excluded from statistical analysis.

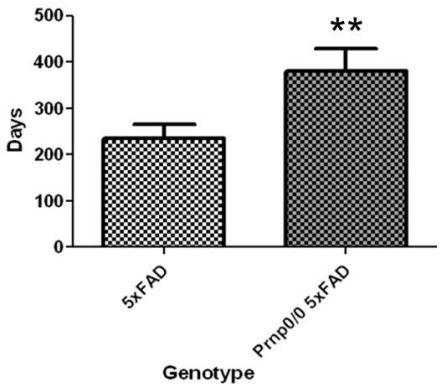

Figure 6. Determination of life span. During a period of two years we monitored the survival of 5xFAD mice. Our data show that $Prnp^{0/0}$ 5xFAD mice have a longer average life span (more than 100 days) than 5xFAD mice. We calculated the p values for statistical analyses. The number of stars

indicates the significance level: one star (*) for p<0.05, two (**) for p<0.01 and three (***) for p<0.001. Values are depicted as mean ± standard error of the mean.

When we compared the life span of 5xFAD mice with Prnp$^{0/0}$5xFAD, we found that PrP^{C} knockout mice (379,3 ± 50,11, n=6) had a longer life span (more than 100 days) than 5xFAD mice (207,0 ± 26,38, n=8) (Figure 6). The *t*-test (p(0.05)= 0,0067) confirmed a significant influence of PrP^{C} in mice life span.

1.2 Deficits in nest building behavior depend on 5xFAD mutation

To assess the impact of 5xFAD mutation on nest building behavior, mice (3, 9 and 12 month-old) were placed individually into a home cage with an untouched sheet of paper tissue (n=6 per group). The next day the quality of the nest was evaluated according to a five-point scale from *Deacon et al* (Deacon 2006). The 3 months old groups (not shown) do not present significant variation in the nest quality among them (WT vs 5xFAD and Prnp$^{0/0}$ vs Prnp$^{0/0}$5xFAD).

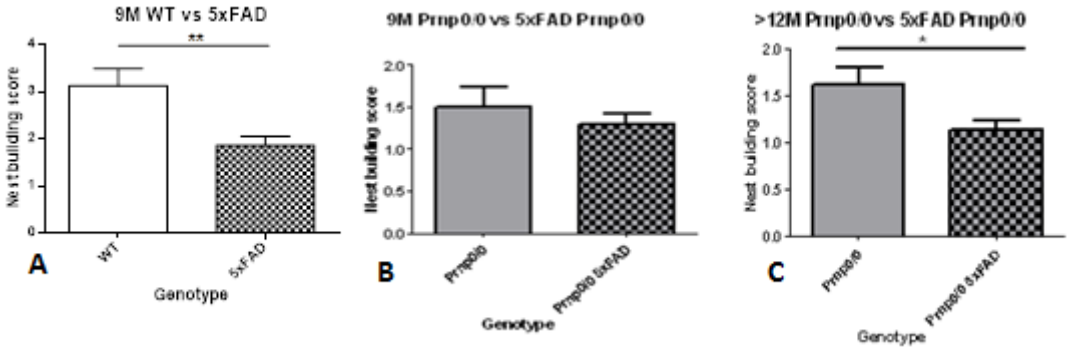

Figure 7. Deficits in nest building behavior of 5xFAD mice. The nest building behavior of WT vs 5xFAD and Prnp$^{0/0}$ vs. Prnp$^{0/0}$5xFAD mice was analyzed after placing a sheet of tissue paper into the cage overnight. The evaluation of nest quality revealed statistically significant differences into A and C groups. Graph B showed no significant difference among Prnp$^{0/0}$ vs. Prnp$^{0/0}$5xFAD (9 months' age). We calculated the p values and the number of stars indicates the significance level: one star (*) for p<0.05, two (**) for p<0.01 and three (***) for p<0.001. Values are depicted as mean ± SEM. **Legend:** (A) 9 months age, WT vs 5xFAD; (B) 9 months age, Prnp$^{0/0}$ vs Prnp$^{0/0}$5xFAD; (C) 12 months age, Prnp$^{0/0}$ vs Prnp$^{0/0}$5xFAD.

In 9 months groups (Figure 7), WT mice built a significantly better nest than 5xFAD. Nevertheless, in 9 months age, Prnp$^{0/0}$ mice built approximately equal nests

as the nests of Prnp$^{0/0}$5xFAD mice, statically there is no significant differences in the nest quality among these two groups. Interestingly, the evaluation of nests from Prnp$^{0/0}$5xFAD (12 months) had a significantly lower nest scores compared with respective control mice (Prnp$^{0/0}$). The evaluation of the nest quality revealed a nesting score which is dependent from the 5xFAD mutations WT mice exhibited an average nesting score of 3, 5xFAD mice of 1.5, Prnp$^{0/0}$5xFAD mice of 1.2 and Prnp$^{0/0}$ mice of 1.5. The observed differences between Prnp$^{0/0}$5xFAD and Prnp$^{0/0}$ are statistically significant after 12 months (Figure 7).

1.3 PrP^{C}-related changes in exploratory and locomotor behavior of 5xFAD mice

In the first behaviour test, we examined the exploratory and locomotor behavior of our different mice groups (WT, 5XFAD, Prnp$^{0/0}$ and Prnp$^{0/0}$5xFAD) by using the open field test (n=6 per group). Mice groups at different ages (3, 9 and 12 months) were allowed to explore a new environment in the open field test for 5 min. Behavioral measures include the total crossing times (locomotor activity and exploratory behavior) and time spent into central area (anxiety).

Animals from all three months old groups exhibited a very similar locomotor activity and anxiety levels during the 5-min session (not shown), no significant differences were found among them. In 9 months old mice groups, WT mice exhibited higher locomotor activity than 5xFAD mice. The t-test ($p< 0.05$) reveals that differences are significant among WT and 5xFAD. Analyzing the data from Prnp$^{0/0}$ and Prnp$^{0/0}$5xFAD mice, we observe identical number of crossing times, Prnp$^{0/0}$ mice (74,00 ± 10,24, n=6) and Prnp$^{0/0}$5xFAD mice (51,00 ± 16,54, n=6) showed no significant ($p>0.05$) locomotor activity. Considering the different areas of open-field, WT mice avoided the central area (high level of anxiety) and 5xFAD spent more time than WT into central area of the OF apparatus. 5xFAD exhibited less anxiety than WT and these results are statistically relevant ($p<0.05$). Interestingly, Prnp$^{0/0}$ and Prnp$^{0/0}$5xFAD mice displayed identical anxiety levels between them ($p>0.05$). However, after 12 months of age, the *t*-test reveals statistical differences between Prnp$^{0/0}$ and Prnp$^{0/0}$5xFAD in locomotor activity ($p<0.05$) and anxiety levels ($p<0.05$).

Prnp^{0}/^{0}5xFAD crossed less times and had less anxiety levels compared with Prnp^{0}/^{0}. The significant results are shown in Figure 8.

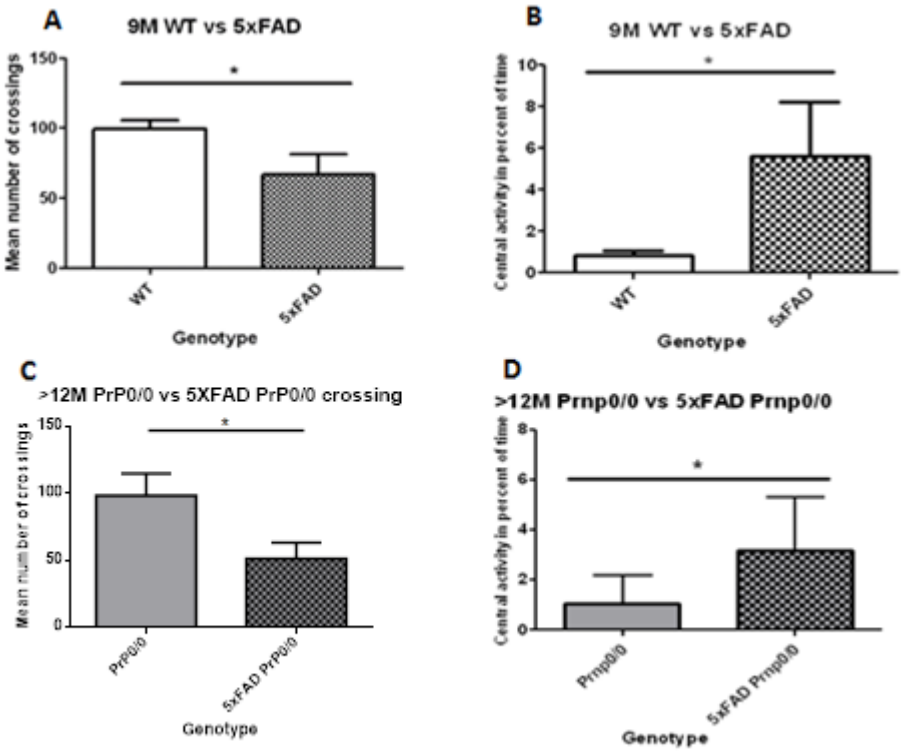

Figure 8. Determination of the exploratory behavior and locomotor activity using the open field test. The number of crossings as well as the activity of the animals in the central and peripheral squares were tallied. The evaluation of exploratory behavior and locomotor activity revealed statistically significant differences only in A and C groups. The relevant differences in anxiety-related behavior were found in B and D groups. We calculated the p values. The number of stars indicates the significance level: one star (*) for p<0.05, two (**) for p<0.01 and three (***) for p<0.001. Values are depicted as mean ± standard error of the mean. **Legend:** (A) locomotor activity, WT vs 5xFAD (9 months age); (B) anxiety-related, Prnp^{0}/^{0} vs Prnp^{0}/^{0}5xFAD (12 months age); (C) locomotor activity behavior, WT vs 5xFAD (9 months age); (D) anxiety-related behavior, Prnp^{0}/^{0} vs Prnp^{0}/^{0}5xFAD (12 months age).

1.4 Object discrimination ability in 5xFAD mice

In the novel object recognition test mice learned to discriminate between a habituated (T_F) and a novel object (T_N). To investigate the impact of PrP^{C} on object discrimination skills we subjected WT, 5xFAD, Prnp^{0}/^{0} and Prnp^{0}/^{0}5xFAD mice at

different ages (3, 9 and 12 months) to the NOR test (n=6 per group). The discrimination ability is indicated is by the *Recognition Index* (RI), i.e., the time that mice spent to investigate the novel object relative to the total number of objects investigated [RI = $T_N/(T_N + T_F)$].

The ability to recognize the new object was similar in all 3 months-old groups (not shown). At 9 months of age 5xFAD mice were less capable than WT mice to discriminate between the habituated object and the new one. The *t*-test ($p<0.05$) confirmed significant differences in RI among WT and 5xFAD (Figure 9A). Data from $Prnp^{0/0}$ and $Prnp^{0/0}$5xFAD (9 months) revealed that both groups were able to discriminate between a previously-encountered object and a novel object ($p<0.05$) (Figure. 9B). After 12 month of age (Figure 9C), in $Prnp^{0/0}$5xFAD mice the new object recognition capacity was decreased compared with $Prnp^{0/0}$. This variation is statistically significant ($p<0.05$).

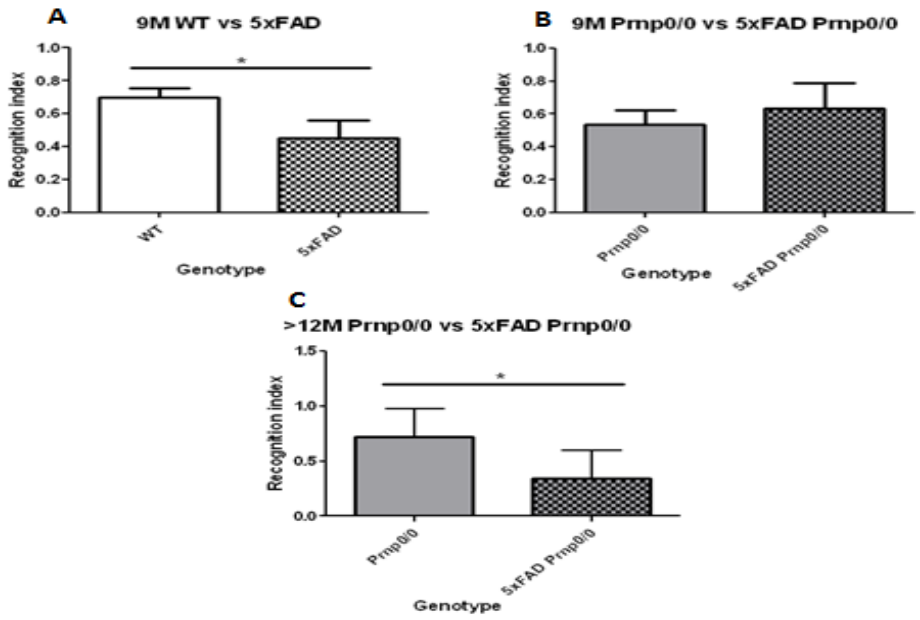

Figure 9. Object discrimination skills. In the NOR test we measured the investigation time of the novel object in relation to the investigation of both objects (RI). Significant statistical differences were found in A and C groups. We calculated the p values and the number of stars indicates the significance level: one star (*) for $p<0.05$, two (**) for $p<0.01$ and three (***) for $p<0.001$. Values are depicted as mean ± standard error of the mean. **Legend:** (A) 9 months age, WT vs 5xFAD; (B) 9 months age, $Prnp^{0/0}$ vs $Prnp^{0/0}$5xFAD; (C) 12 months age, $Prnp^{0/0}$ vs $Prnp^{0/0}$5xFAD.

1.5 Effect of PrP^C level on the general anxiety behaviour of 5xFAD mice

Elevated plus-maze task allowed us to analyze the general anxiety, which can be indicated by the time spent on the open arms. This test is based on the conflict between the natural desire of mice to explore a new environment and their fear of open and high spaces. The more time mice spend into open arms less general anxiety they experience.

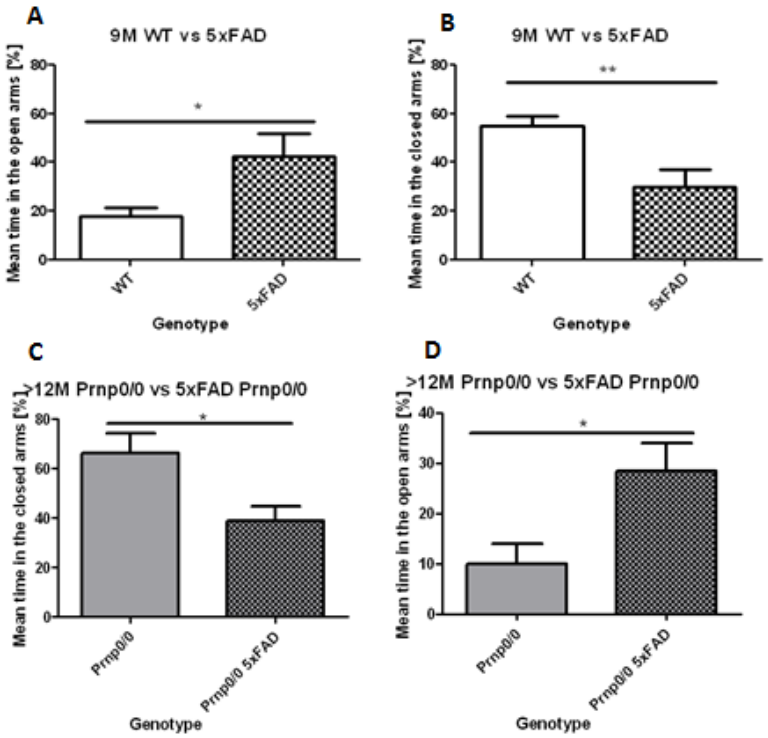

Figure 10. Basal anxiety in 5xFAD mice is dependent on PrP level during aging. The elevated plus maze test revealed a decrease in general anxiety behavior (duration in open arms) in 9 months-old 5xFAD mice compared to WT mice of the same age (A and B). No differences could be observed between $Prnp^{0/0}$5xFAD and $Prnp^{0/0}$ mice after 9 months of age. After 12 months of age we observe that general anxiety decreased in $Prnp^{0/0}$5xFAD mice compared to $Prnp^{0/0}$ controls without amyloid mutation (C and D). We calculated the p values. The number of stars indicates the significance level: one star (*) for $p<0.05$, two (**) for $p<0.01$ and three (***) for $p<0.001$. Values are depicted as mean ± standard error of the mean. **Legend:** (A) Mean time on open arms, WT vs 5xFAD (9 months age); (B) Mean time into closed arms, WT vs 5xFAD (9months age); (C) Mean time into closed arms, $Prnp^{0/0}$ vs $Prnp^{0/0}$5xFAD (12 months' age); (D) Mean time on open arms, $Prnp^{0/0}$ vs $Prnp^{0/0}$5xFAD (12 months' age).

We used single exposure to the elevated plus maze to determine anxiety behavior of mice. The four groups (n=6 per group) were tested. Three month old mice showed similar anxiety levels (not shown). As illustrated in Figure 10, there is a

clear difference between mouse groups in general anxiety after 9 months age. Wild type group stayed longer in close arms (Figure 10A) and less time in open arms (Figure 10B) than 5xFAD. The t-test of time spent in closed arms was ($p<0.01$). The interpretation of these results reveals a less anxious behavior of 5xFAD mice compared to WT mice. $Prnp^{0/0}$ and $Prnp^{0/0}$ 5xFAD (9 months showed no significant differences of anxious behavior between them. The *t*-test confirmed the similar behavior ($p> 0.05$) between $Prnp^{0/0}$ and $Prnp^{0/0}$5xFAD, closed arms *t*-test and open arms t-test. Statistically significant results were observed among $Prnp^{0/0}$ and $Prnp^{0/0}$5xFAD after 12 months of age. The $Prnp^{0/0}$ spent less time than $Prnp^{0/0}$5xFAD in open arms ($p<0.05$). $Prnp^{0/0}$5xFAD had less anxiety than $Prnp^{0/0}$ (Figure 10C and 10D).

1.6 Effect of PrP^{C} level on motor performance of 5xFAD mice

In experiment we assessed motor performance using the rotarod test. Motor performance was determined by measuring the time spent on the rotating rod (n=6 per group). The 12-month-old groups were analyzed in a separate trial, in which the spinning of the rod was adapted to mouse age.

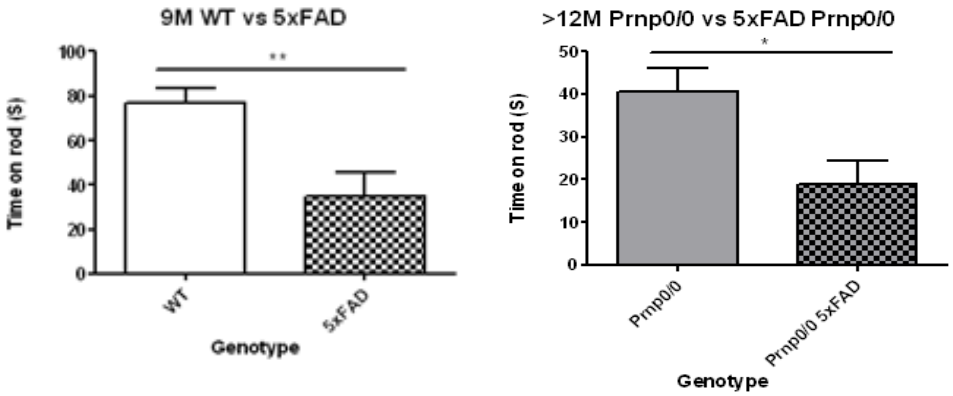

Figure 11- Motor performance. In the rotarod task we determined the time, which the mice spent on the rotating rod. 5xFAD mice (9 months old) showed early motor impairment than 5xFAD $Prnp^{0/0}$ (12 months old). We calculated the p values. The number of stars indicates the significance level: one star (*) for $p<0.05$, two (**) for $p<0.01$ and three (***) for $p<0.001$. Values are depicted as mean ± standard error of the mean.

All 3 months of age groups exhibited a good performance and no motor significant differences were found between WT and 5xFAD or Prnp$^{0/0}$ and Prnp$^{0/0}$5xFAD (not shown).Differences ($p<0.01$) started to appear between WT and 5xFAD at 9 months, the WT group performed longer than 5xFAD, this means that WT had better motor skills than 5xFAD.The rotarod performance was not significantly changed among Prnp$^{0/0}$ and Prnp$^{0/0}$5xFAD ($p>0.05$). Differences occurred between Prnp$^{0/0}$ and 5xFAD Prnp$^{0/0}$ for older mice (12 months) ($p<0.05$). 5xFAD Prnp$^{0/0}$ mice were less capable to walk on rotarod compared with Prnp$^{0/0}$. The statistical relevant results are presented in Figure 11.

1.7 Effect of PrP^{C} level on associated learning and memory skills of 5xFAD mice

To investigate the impact of PrP^{C} level on the associative learning and memory skills of 5xFAD mice we subjected mice to the fear conditioning test. Freezing response will be demonstrated if the mouse remembers the environment with aversive stimulus. All mice were tested 24h later in the conditioning chamber for contextual learning (n=6 per group).

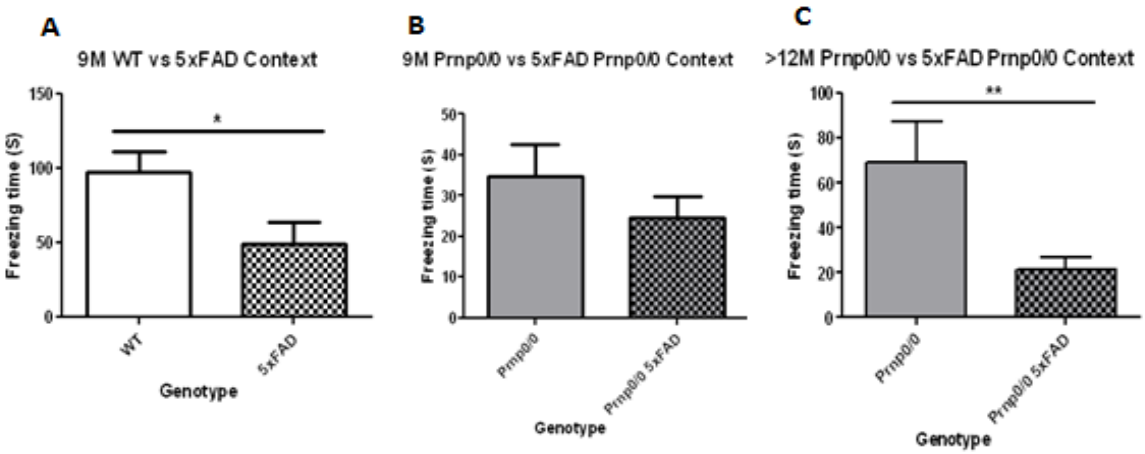

Figure 12 - Contextual learning. Twenty-four hours after they received the footshock, the mice were monitored and it was quantified the freezing time. We calculated the p values. The t-test showed significant differences into 5xFAD (9 months old) mice group (A) and into Prnp$^{0/0}$5xFAD (12 months old) mice group (C). The number of stars indicates the significance level: one star (*) for $p<0.05$, two (**) for $p<0.01$ and three (***) for $p<0.001$. Values are depicted as mean ± standard error of the mean.

As expected, all 3 months old groups behaved in a similar manner (not shown), they were able to recognize the context of the aversive stimulus. In contextual test of the 9 months old groups, the *t*-test ($p<0.05$) showed significant differences between WT and 5xFAD. The majority of 5xFAD mice were not capable to link the context of the aversive stimulus (Figure 12A). In contrast, differences between $Prnp^{0}/^{0}$ and $Prnp^{0}/^{0}$5xFAD freezing time was not statistically significant (Figure 12B), the performance was similar ($p>0.05$). The major differences ($p<0.01$) were observed among $Prnp^{0}/^{0}$ and $Prnp^{0}/^{0}$5xFAD, the later ones showed a higher memory impairment compared with control group (Figure 12C).

2. Molecular results

2.1 Effect of PrP^C presence/absence on the Aβ production levels in 5xFAD mice

At first we examined the levels of $Aβ_{1-40}$ and $Aβ_{1-42}$ in the brain tissue of different mice groups (WT, 5xFAD, $Prnp^{0/0}$ and $Prnp^{0/0}$5xFAD at different ages (3 and 9 months) via ELISA (Figure.5). We found the levels of $Aβ_{1-40}$ and $Aβ_{1-42}$ was markedly increases in 5xFAD mice due to the familial AD mutations, independently from the expression of PrP^C. During aging (between 3 and 9 months) both amyloid forms increased dramatically (more than 3 times).

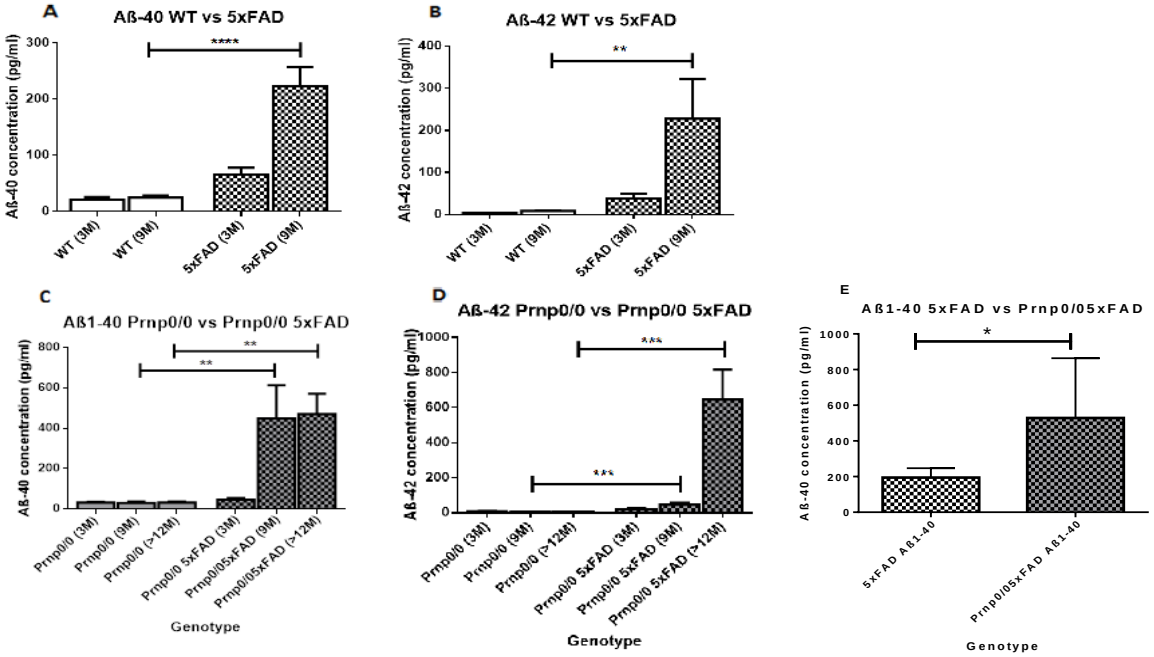

Figure 13. Effect of PrP^C level on the amount of Aβ1-40 and Aβ1-42 in transgenic mice. Concentrations of $Aβ_{1-40}$ (6A and 6C) and $Aβ_{1-42}$ (6B and 7D) were determined in WT, 5xFAD, $Prnp^{0/0}$ and $Prnp^{0/0}$5xFAD mice at an age of 3 and 9 months via IBL-ELISA. Levels of $Aβ_{1-40}$ and $Aβ_{1-42}$ were increased after 9 months. For comparison between groups we used the student's t-test (n=8 per group in three independent experiments). Error bars indicate standard deviation of the mean (SEM). One star (*) for p<0.05, two (**) for p<0.01, three (***) for p<0.001 and four (****) for p<0.0001. Values are depicted as mean ± standard error of the mean.

2.2 Immunohistochemical staining analysis of Aβ plaque deposits in 5xFAD

To investigate the impact of PrP^C on the localization of Aβ plaques we performed an immuno staining of brain tissue derived from 9 months old mice. The figures show a diffuse distribution of Aβ plaques in the total brain tissues in both, 5xFAD (Figure 7C) and $Prnp^{0/0}$5xFAD (Figure 7D). Control animals without 5xFAD mutation had no Aβ plaques in their brains (Figure 7A and 7B). No differences regarding the amount of Aβ deposits could be observed in both 5xFAD and $Prnp^{0/0}$5xFAD mouse lines (Fig. 7C and 7D).

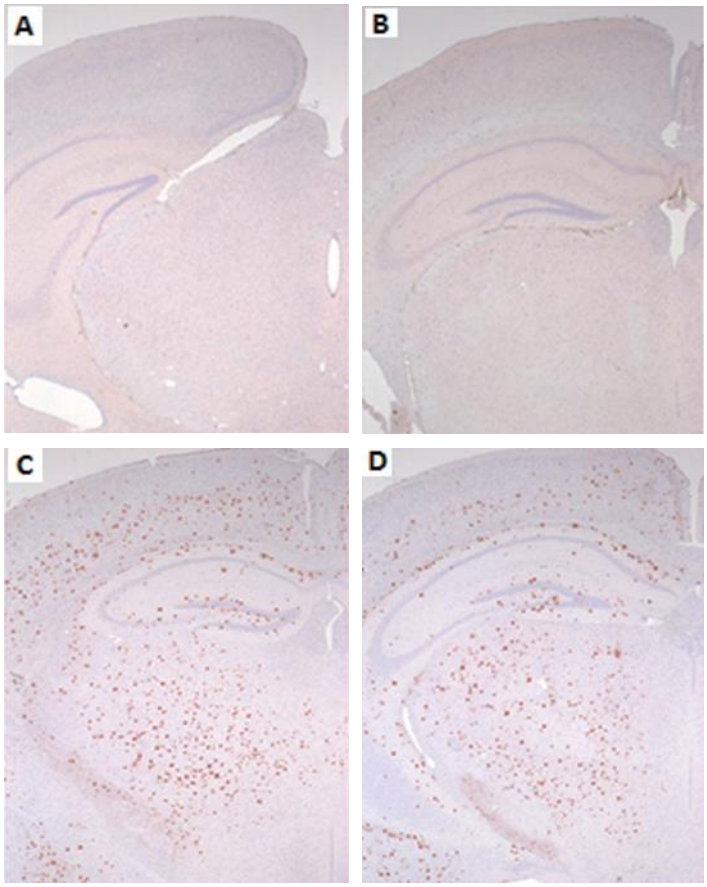

Figure 14. Immunohistochemical staining of Aβ plaque deposits in 9 month-old 5xFAD mice. DAPI-staining shows the dentate gyrus. No amyloid plaques were observed in WT (A) or $Prnp^{0/0}$ (B). In 5xFAD (C) and $Prnp^{0/0}$5xFAD (D) mice the localization of plaques is diffuse all over the brain. The number of plaques was not significantly different in both 5xFAD mice groups (C and D).

2.3 Role of PrP^C presence/absence in cell signaling

To determine the role of PrP^C in cell signaling we performed western blot analysis. We searched for Fyn, P-Fyn and Caveolin 1 expression from cortex of mice (9 months age).

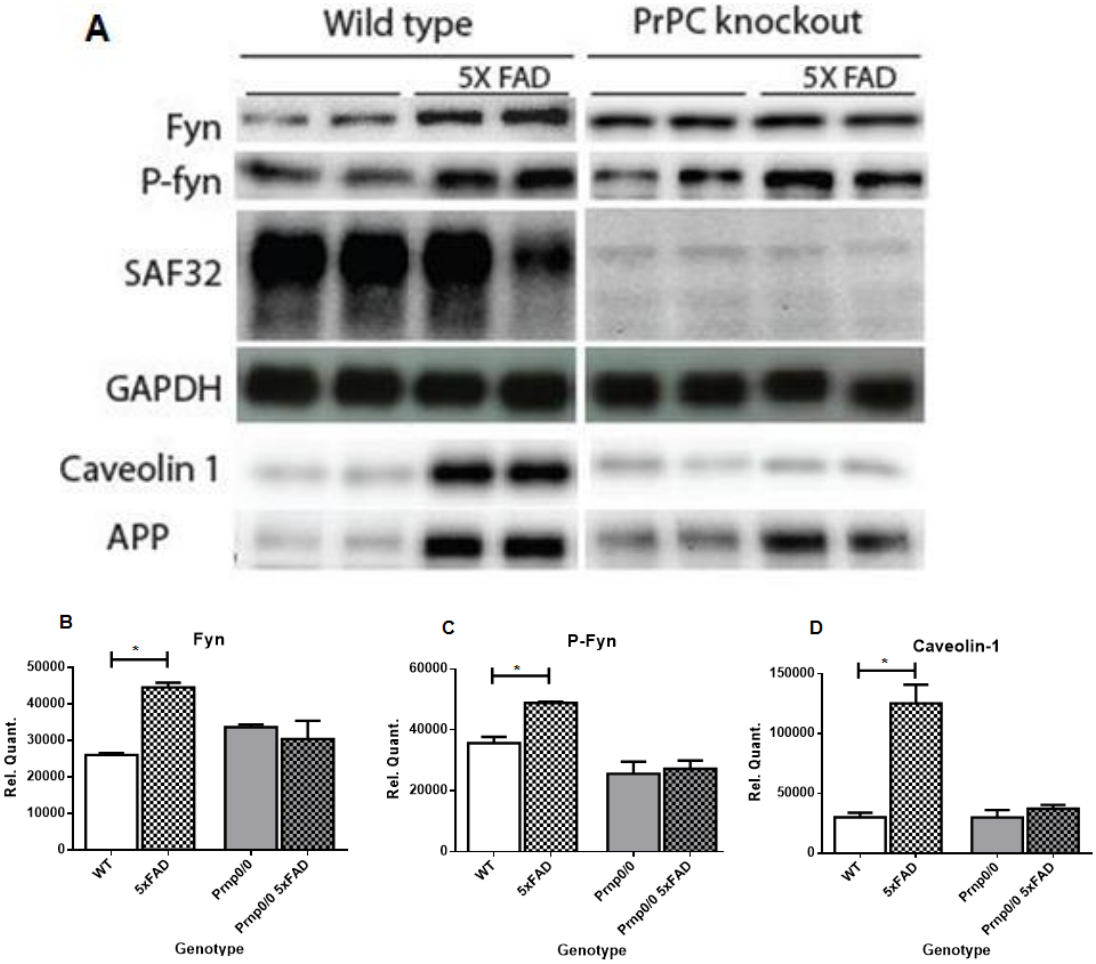

Figure 15. Western blotting from 9 months old groups (Fig. 15A) and relative quantification of bands (Fig. 15.B, 15C and 15D).The results from WB showed an increased amount of Fyn (Fig. 15B), P-Fyn (Fig.15C) and Caveolin 1 (Fig. 15D) in 5xFAD (wild type for PrP^C) mice comparing to WT. SAF32 and APP confirmed the presence of PrP^C expression and 5xFAD mutation, respectively. PrP^C knockout with 5xFAD mutation ($Prnp^{0/0}$5xFAD mice) do not exhibit a different expression pattern of Fyn, P-Fyn and Caveolin 1 comparing to PrP^C knockout without 5xFAD mutation ($Prnp^{0/0}$) (Fig. 15.B, 15C and 15D). SAF32 antibody was used to confirm the absence of PrP^C in PrP^C knockout group. APP antibody proved the 5xFAD mutation in PrP^C knockout group ($Prnp^{0/0}$ 5xFAD). GAPDH antibody present the same protein amount in all samples, it means the differences seen in WB results are not due to different proteins amount (Fig.15A).

These proteins are known to be involved in Alzheimer disease pathway. 5xFAD mice (9 months age) showed (Figure 15), as expected, an increased expression of Fyn, P-Fyn and Caveolin 1 comparing to WT mice. We did not noticed a different expression pattern of these three proteins in $Prnp^{0/0}$5xFAD comparing to $Prnp^{0/0}$ and the expression of Fyn, P-Fyn and Caveolin 1 in $Prnp^{0/0}$5xFAD is nearly the same as in $Prnp^{0/0}$. GAPDH antibody was used as loading control (protein amount), SAF-32 antibody was the control for PrP^{C} expression (presence/absence) and APP antibody was used as a 5xFAD mutation control.

3. Genotyping

All mice were genotyped after birth by PCR. A biopsy from tail was used to check the genotype after sacrifice. $Prnp^{+/+}$, $Prnp^{0/0}$ and APP mutation (5xFAD) were analysed.The figure 16 shows the representative results from PCRs.

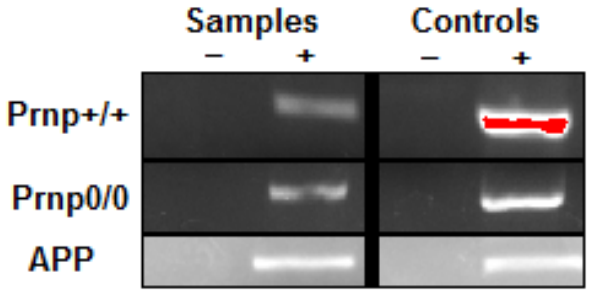

Figure 16. Mice's genotype. The image shows the positive and negative results for each genotype with their respective controls.

V. Discussion

The major defined pathological hallmark of Alzheimer's disease is the accumulation of amyloid beta, a neurotoxic peptide derived from beta and gamma-secretase cleavage of the amyloid precursor protein. In this project we intend to investigate the role of the cellular prion protein (PrP^C) in AD which is still contradictious. To clarify this issue we used two transgenic mice (5xFAD and $Prnp^{0/0}$5xFAD) by crossing PrP^C knockout ($Prnp^{0/0}$) mice (described by Büeler *et al.*(1992)) and 5xFAD mice exhibiting mutations in the APP and presenilin genes. The 5xFAD mice (Tg6799) genetic background C57Bl/6J, have been previously described (Oakley et al. 2006). The 5xFAD mouse is one of a few known mouse models that exhibits significant neuron loss in addition of displaying other AD hallmarks such as amyloid plaques (Guglielmotto et al. 2012; Casas et al. 2004; Oakley et al. 2006). These mice express the 695 aminoacids isoform of the human amyloid precursor protein (APP695) carrying the Swedish/London/Florida mutations under the control of the murine Thy-1-promoter. In addition, human presenilin-1 carrying the M146L/L286V mutations is expressed also under the control of the murine Thy-1-promoter. In these mice, the potential behavior changes at different ages by using a battery of standardized behaviour tasks was analysed. Moreover the progression of the amyloid pathology and the amount of Aβ in both mouse models were analysed in dependence of the PrP^C expression via immunohistochemical staining and ELISA analysis. From these data we evaluated the potential of PrP^C as a positive/negative key-player in AD and its potential as a therapeutic target.

1. Role of PrP^C in AD

In addition to its protective function under oxidative stress conditions (Brown et al. 1997; Ramljak et al. 2015) increasing evidence suggest that PrP may play a role in Alzheimer's disease or during the development of AD- pathology:

a) PrP^C co-localizes with amyloid plaques (Schwarze-Eicker et al. 2005; Takahashi et al. 2011) which are present in CJD patients with amyloid pathology (Del Bo et al. 2006).

b) The codon 129 methionine/valine polymorphism of the human *PRNP* influences the susceptibility to prion diseases. Furthermore, certain amino acid variations may also be a risk factor for early onset of AD (Dermaut et al. 2003; Riemenschneider et al. 2004) and the codon 129 polymorphism can modulate the number of amyloid deposits during cerebral aging (Berr et al. 2003).

c) In disease models, clear evidence exists that PrP^C is involved in the pathophysiological metabolism of APP. PrP^C contains an amyloid oligomer binding site, a region within the central part of PrP^C from amino acids 95-110 (Laurén et al. 2009). PrP^C thus may act as a potential receptor for Aβ mediating the toxicity of Aβ-oligomers.

Nevertheless, three independent studies failed to confirm the postulated PrP^C-promoted toxic effects (Balducci et al. 2010) leaving the debate on the role of PrP^C still open.

2. Influence of PrP^C on Aβ production

Aβ is formed during the amyloidogenic pathway of APP processing by cleavage of APP by two secretases, the beta-secretase and the gamma-secretase. Recent studies on AD models indicated a causal involvement of PrP^C in the amyloid pathogenesis of AD. An in vitro study indicated that PrP^C may influence the processing of APP by inhibition of BACE1 (Parkin et al. 2007) and it was proposed that one of the physiological functions of PrP^C may be the regulation of the β-secretase activity.

To investigate the role of PrP^C on the processing of APP we examined the expression of $Aβ_{1-40}$ and $Aβ_{1-42}$ in brain tissue of different mice groups (WT, 5xFADWT, $Prnp^{0/0}$ and $Prnp^{0/0}$5xFAD) at different ages (3, 9 and 12 months) via ELISA. We found that levels of $Aβ_{1-40}$ and $Aβ_{1-42}$ was, as expected, dramatically up-

regulated in 5xFAD mice independently from PrP^C. During aging process (between 3 and 9 months) levels of both amyloid forms increased markedly, which are induced by the familial AD mutations. When we compared the amount of Aβ in 5xFAD and $Prnp^{0/0}$5xFAD, we found a significant increase of $Aβ_{1-40}$ but not the pathogenic form, $Aβ_{1-42}$, in $Prnp^{0/0}$5xFAD. Our results suggest that PrP^C may influence $Aβ_{1-40}$ amount but, apparently, it does not influence the $Aβ_{1-42}$ generation. Our findings agree in part with a previous in vitro study describing that PrP^C affects Aβ levels by decreasing the cleavage of APP by BACE1. PrP^C can influence the processing of APP and the amount of APP-cleavage products $Aβ_{1-40}$ and $Aβ_{1-42}$, while the expression of APP and β-secretase remained unchanged, which is in line with others authors (Gimbel et al. 2010; Parkin et al. 2007).

One explanation for the selective regulation of $Aβ_{1-40}$ but not of $Aβ_{1-42}$ via PrP^C may be that APP metabolism is more complex, e.g. APP can be processed by different pathways. Not all were influenced by PrP^C. Another possibility is via protein binding. Previous studies revealed that PrP^C can potentially interact with APP and BACE. While PrP^C interaction with the pro-domain of an immature Golgi-associated form of BACE1 may restrain BACE1 in the trans-Golgi network, (Gimbel et al. 2010), PrP^C might additionally influence the APP cleavage via binding to APP/sAPP (Schmitz et al., 2014). This inhibition might be explained by conformational changes within full-length APP which may, at least partly, bury the binding epitope of PrP^C (Schmitz et al., 2014).

Altogether, the fact that PrP^C reduces the amount of cell-toxic $Aβ_{1-40}$ but not $Aβ_{1-42}$ may indicate that PrP^C may have a neuroprotective function in AD. To elucidate the potential role of PrP in AD further studies on the interaction of PrP an Aβ and on the influence of PrP on the Aβ induced toxicity were undertaken.

3. Cellular prion protein do not influence the distribution of β-amyloid deposition in 5xFAD mice

Amyloid plaques, deposits of the Aβ peptide (Glenner & Wong 1984), are defining lesions in AD brain. β-secretase has been implicated in amyloid production and plaque formation (Cai et al. 2012), and its activity is directly related to Aβ load

(Capetillo-Zarate et al. 2012; Ho et al. 2005). Additionally, it has been proposed that PrP^C expression may decrease plaque formation (Chung et al. 2010). We performed an immunohistochemical staining of Aβ deposits in brains derived from 5xFAD and $Prnp^{0/0}$5xFAD mice. We showed in both mice models a diffuse distribution of Aβ deposits, whose amount is increased during the aging process. However, a comparison of the distribution and number of Aβ deposits revealed no significant differences between both mice models. Several authors have proposed that APP is cleaved by BACE1, and BACE1 is inhibited by PrP^C, wherefore the absence of PrP^C should promote an increase in Aβ production, supporting a relationship between BACE1 and PrP^C (Kellett & Hooper 2009; Parkin et al. 2007). However, most of these studies were performed in cell culture. A study performed in an AD mouse model, indicated that the deletion of PrP^C did not affect APP or Aβ deposition (Griffiths et al. 2011). According to our results, it may be speculated that instead basal amyloid levels are modified, secondary events associated with alternative production of amyloid and/or degradation events influence Aβ deposition (Ordóñez-Gutiérrez et al. 2013), for instance, disruption of lysosomal proteolysis and autophagy has been described in response to Presenilin 1 mutation. Beyond what was previously described, it has been suggested that Aβ undergo phosphorylation. The phosphorylation may lead the conformational transition and formation of toxic aggregates. Additionally, phosphorylated Aβ seems to be resistant against degradation by proteases (Kumar & Walter 2011).

4. $Prnp^{0/0}$5xFAD mice exhibited a longer life span and a delayed occurrence of behavioral deficits

Alzheimer's disease is characterized by a deficit in motor and spatial learning–memory and alteration of non-cognitive behavior. Lauren *et al.* (2009), found that Aβ oligomers inhibit long-term potentiation (LTP) in hippocampal slices from normal mice, but not in hippocampal slices from mice lacking PrP^C. LTP provides a measure of synaptic plasticity related to learning and memory, these two skills are compromised in Alzheimer's disease. As mentioned before, our intention is to rule out PrP^C related effects in our behavior study (analyzed before by Schmitz et al.,

2014) and to focus on Aβ-induced deficits in mice solely. We analyzed 5xFAD mice in comparison to WT and compared $Prnp^{0/0}$5xFADmice to $Prnp^{0/0}$ animals. To understand the role of PrP^{C} in AD we monitored the survival of animals over a 2 years period and we used a panel of different behavioral tests (motor function, general anxiety behavior, and cognition) to assess the onset and progression of Aβ-induced deficits, in presence/absence of PrP^{C}.

Basal anxiety and exploratory behavior in mice were investigated using the EPM and open field tests, respectively. The basal anxiety was significantly reduced paired with a decreased locomotion and exploration in 5xFAD mice compared to WT animals at 9 months of age. In the literature, anxiety-related behavior has been demonstrated to be reduced in different AD mice models (Jawhar et al. 2012; Lalonde et al. 2003; Faure et al. 2011). The impairment may be interpreted in many ways, for instance, a loss in motivation to explore, inhibitory control or spatial orientation (Lalonde 2002), similar to symptoms described in Alzheimer dementia (Chung & Cummings 2000; Frisoni et al. 1999; Daffner et al. 1992). Interestingly, in absence of PrP^{C}, the behavior of the mice ($Prnp^{0/0}$5xFAD 9 months old) seems to be not affected by the high levels of β amyloid. Although later (12 months old) $Prnp^{0/0}$ 5xFAD mice showed a decreased anxiety and less locomotor activity when compared with $Prnp^{0/0}$ 12 months old group. Similar results were found in learning and memory tests. To investigate learning and memory deficits, the cardinal symptoms of AD, we performed the Fear Conditioning and NOR tests. We demonstrated significant memory deficits in 9 months old 5xFAD mice. Our results are in agreement with several studies demonstrating reduced working memory levels that decline with age in several of AD models (Chapman et al. 1999; Lovasic et al. 2005; Duyckaerts et al. 2008; Wirths et al. 2008). A similar impairment was found in our results (in 5xFAD $Prnp^{0/0}$ 12 months old mice). Both results suggest a decline of associative learning and memory. It has been postulated that the decline of memory function begins around 6 months of age in 5xFAD mice (Devi & Ohno 2010). However, these deficits were not observed until 12 months old in $Prnp^{0/0}$5xFAD mice.

We used rotarod performance which requires postural adjustments for the maintenance of equilibrium on a rotating and accelerating rod. We observed an age-dependent decrease in motor function only at 9 month old in 5xFAD mice and at 12

months old in Prnp^{0}/^{0}5xFAD. Prnp^{0}/^{0}5xFAD (12 months of age) showed less ability to performed rotarod test when compared to their controls. The ability to perform the rotarod task cannot be attributed to a single brain region, it has been shown to be a sensitive indicator of cerebellar abnormalities, for example cerebellar cortex, deep cerebellar nuclei, dysfunction and/or neuronal loss (Hilber & Caston 2001).

The nest provides them, among other things, shelter, heat conservation and reproduction. It is essential for long lifetime and reproductive success of mice (Bult & Lynch 1997). 5xFAD 9 months old mice were less capable and less motivated to build their nest when compared with the same age wild type group, resulting in very poorly shredded, almost untouched material. Poor nest build was reported in other mice with AD mutations (Filali et al. 2009; Deacon et al. 2008). Nesting behavior is reduced along age, paralleling with increase of Aβ burden with age (Min et al. 2013). However, it seems that this tendency is quite retarded in Prnp^{0}/^{0}5xFAD. Our results show that the deficits found in 5xFAD mice (9 months) are delayed in Prnp^{0}/^{0}5xFAD, since for the later these deficits only appear at 12 months age.

Alongside behavioral tests we recorded the mortality of the mice. Interestingly, the analysis 5xFAD exhibited a significant shorter life span (almost 50% less) than Prnp^{0}/^{0}5xFAD mice, indicating a clear effect of PrP^{C} on the survival of 5xFAD mice. Due to this reason, we have not tested any group more than 12 months.

Overall, as expected, in all behavioral tests the 5xFAD mice showed a significant decrease of their performance potential due to Aβ toxicity. Aβ peptide is formed extracellularly and to induce toxicity it is necessary that the toxic signal pass through the membrane. PrP^{C} has been suggested as a Aβ receptor inducing Aβ toxicity into cell (Laurén et al. 2009). This hypothesis may explain the decrease of performance in 5xFAD mice during aging. Although, in the absence of PrP^{C} (Prnp^{0}/^{0}5xFAD mice), deficiencies also appear, they are delayed. The delayed impairments might be due to the presence of other receptors for Aβ besides PrP^{C}. Other proteins have been described as “Aβ receptors”, for instance, *N*-methyl-D-aspartate receptors (NMDARs), glycation end products (RAGE), α7-nicotinic acetylcholine receptor (α7 nAChR), ephrin type B receptor 2, immunoglobulin G Fc gamma receptor IIb (FcγRIIb), and paired immunoglobulin-like receptor B (PirB) (reviewed in Kam, Gwon and Jung, 2014). Undoubtedly, PrP^{C} may play a main role in

inducing Aβ neurotoxicity, but it seems not to be the only one. In the absence of PrP^C, it may be required a greater amount of Aβ for the manifestation of the disease.

5. Regulation of downstream signaling proteins via PrP^C inducing the toxicity of Aβ

It was suggested that PrP^C mediates the toxic effect of Aβ oligomers and is required for Aβ oligomer-induced suppression of synaptic plasticity, synapse damage, and neuronal cell death (Laurén et al. 2009; Bate & Williams 2011). Several reports showed that oligomeric amyloid-β binds to residues 23-27 and 95-110 of PrP^C, which underlines the relevance of PrP^C in AD (Chen et al. 2010; Zou et al. 2011; Fluharty et al. 2013; Younan et al. 2013). This binding results in activation of the Fyn kinase (Um et al. 2012). Fyn belongs to the family of Src kinases which are generally involved in signal transduction events (Toni et al. 2006). Activation of Fyn by the Aβ–PrP^C complexes also leads to abnormal hyperphosphorylation of Tau. Fyn activity, like that of other Src family kinases, is regulated by intramolecular interactions that depend on the equilibrium between tyrosine phosphorylation and dephosphorylation (Figure 17). Our western blot analysis (from 9 months old mice) revealed an up-regulation of Fyn and P-Fyn levels (in mice with AD mutation), due to the presence of PrP^C, since in the absence of PrP^C ($Prnp^{0/0}$5xFAD mice) there is no up-regulation of both proteins when compared with $Pnrp^{0/0}$. Our results, are in accordance with a previous study which demonstrated that PrP^C may promote Fyn activation when PrP^C bound to Aβ (Laurén et al. 2009). Although, PrP^C (mostly extracellular protein) and Fyn (intracellular protein) are differentially located within cells which prevents a direct physical contact (Um et al. 2012), the physiological connection of both proteins suggest the involvement of other signaling proteins (Figure 16).

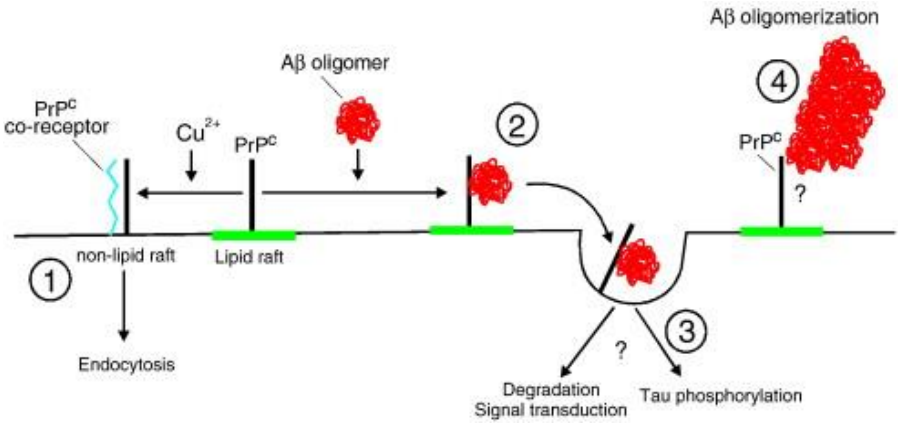

Figure 17. Scheme illustrating the recently reported data on, PrP^C and Aβ oligomer removal. (1) Normal trafficking of the PrP^C from lipid rafts into the cell his mediated by Cu^{2+} and a co-receptor (Taylor & Hooper 2007). Interaction with Aβ oligomers (2) may modify the interaction of PrP^C with its co-receptor leading to a putative endocytic process (3) (Laurén et al. 2009). However, whether this process takes place and Aβ oligomers are degraded or whether it modifies signal transduction as indicated warrant further study. In addition, a link between these interactions and tau phosphorylation is missing. Lastly, a putative interaction of PrP^C with Aβ oligomers to enhance fibril formation and Aβ deposition could take place (4). (Nicolas et al. 2009)

Caveolae are specialized membrane microdomains that contain members of the caveolin protein family. They are enriched in cholesterol and glycosphingolipids and often appear as flask-shaped invaginations in the plasma membrane (Parton, 2003). It has been observed that PrP^C is mainly localized in caveolae membrane domains of neuronal cells (Massimino et al. 2002). It was confirmed that PrP^C is internalized by caveolae (Peters et al. 2003), facilitating internalization of Aβ oligomers into intracellular compartments (Figure 18). The obtained data revealed an up-regulation of Caveolin-1 (Cav-1) in 5xFAD mice in comparison to wild type mice. This up-regulation in AD was ascribed to alteration in membrane properties of brain cells. AD brain have a narrower membrane than normal brain (Gaudreault et al. 2004). Our observation that $Prnp^{0/0}$5xFAD mice did not exhibit up-regulated Cav-1 levels, suggests that regulation of Cav-1 levels is dependent on PrP^C concentration.

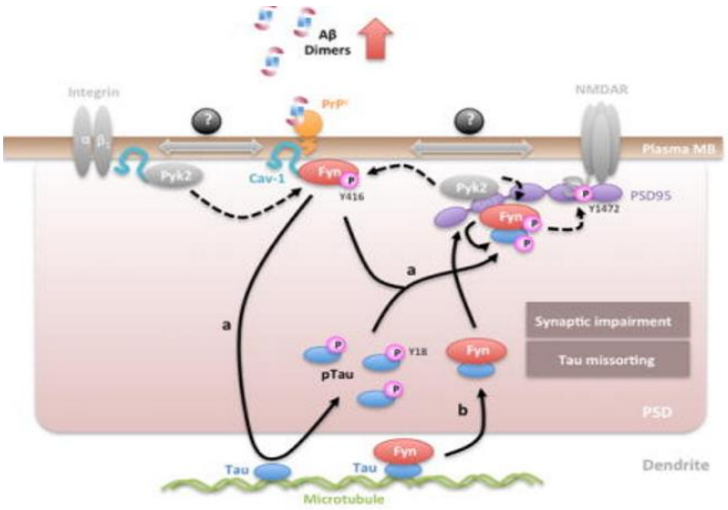

Figure 18. Proposed model of tau regulation by the triad Aβ-PrP^{C}-Fyn. In the presence of accumulating Aβ, PrP^{C}, Fyn and Cav-1 form a complex at the plasma membrane. Upon phosphorylation of Fyn at Y416, this complex becomes biologically active. Two scenarios are possible depending on the status of Fyn with respect to tau: (a), activated Fyn causes the hyperphosphorylation of tau at Y18 and its aberrant accumulation at the postsynaptic density (PSD). In the model (b), Fyn is already bound to tau in the dendrite, translocate to the PSD to interact with PSD95. There, Fyn could be phosphorylated, resulting in Fyn activation and tau phosphorylation at Y18. Adapted from: (Larson et al. 2012)

Excessive activation of NMDARs has also been implicated in AD by engagement of an Aβ/PrP^{C}/Fyn pathway (Paoletti et al. 2013). NMDAR hyperactivity leads to excessive Ca^{2+} influx through NMDARs, ultimately leading to neuronal death. Therefore, it seems that PrP^{C} is crucial for cell damage by Aβ toxicity.

Altogether, we did not found in Prnp^{0}/^{0}5xFAD mice the up-regulation of P-Fyn, Fyn or Cav-1 as we found in 5xFAD mice. This suggests an important role of PrP^{C} in Alzheimer's disease as a promoter of toxic effect of Aβ oligomers. The presence of PrP^{C} seems to have a whole set of circumstances that promote the toxicity of Aβ oligomers, for instance, overregulation of Fyn that is thought to be responsible for Aβ toxicity. However, this does not mean that absence of PrP^{C} can completely prevent the Aβ induced toxicity in cells. These results are still preliminary, more research is required.

VI. Conclusion

Alzheimer's disease is a progressive neurodegenerative disorder that affects millions of people worldwide, although currently has no effective treatment options. It is urgent to know the mechanisms of disease and potential treatments. PrP^C was suggested as a potential target for the treatment of AD due to its important role in the disease. Our study indicated a regulatory effect of the PrP^C level on the processing of APP. Investigating the amount of amyloid beta in 5xFAD mice we observed a PrP^C dependent regulation in 9 month-old animals of $A\beta_{1-40}$ but not of the toxic form $A\beta_{1-42}$. The behaviour study revealed significant deficits in general anxiety, learning performance and motoric function in 5xFAD mice, which correlate with the increase of amyloid beta mediated toxicity during the aging process of 5xFAD mice. Interestingly, behavioural deficits occurred earlier (after 9 months) in 5xFAD mice than in $Prnp^{0/0}$5xFAD (after 12 months) mice indicating that PrP^C may accelerate the amyloid beta-induced toxicity. Our data support a role of PrP^C as accelerator in AD mediating the toxicity of Aβ. In this sense, we can conclude, although does not avoid the disease, PrP^C may be used as a first approach to delay the symptomatic effects of AD.

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